ANNUAL REPORT OF PROGRAM ACTIVITIES NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES FISCAL YEAR 1974

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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Na... of Health
Building 10
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National Institute of Arthritis, Metabolism, and
" Digestive Diseases
Report of program activities

ANNUAL REPORT

OF

PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

Fiscal Year 1974

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National Institute of Arthritis, Metabolism, and Digestive Diseases

Fiscal Year 1974

by

Dr. J. E. Rall

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The National Institute of Arthritis, Metabolism, and Digestive Diseases has continued intramurally research at the most basic level as well as clinical and applied studies. The following report lists in broad outline the major studies which have been carried out in the past year. A few of the more dramatic results can be mentioned and have some implications both for biology directly and for the direction of biomedical research. I would refer to the elegant model provided by x-ray diffraction analysis of a myeloma protein. This shows the precise position of the antigen in the antigen binding site of the antibody as well as those amino acid residues which interact with the antigen. Also to be mentioned are the several studies on chromatin going on in different laboratories of the Institute. These studies have already begun to show how the nucleus is put together, what holds the DNA together, and some beginning insight into how the controls are exerted on the various metabolic processes. Since in eukaryotic cells, including man, it is in general the nucleus which controls all of the metabolic activities and in many instances is responsible in one way or another for the diseases man is heir to, quite clearly, an intimate understanding of the mechanism by which genetic information in the nucleus is carried to the cell and affects metabolic processes is of prime importance.

Another area which is being explored in a wide variety of laboratories in this Institute is the nature of receptors on cell surfaces. This again involves cell surfaces of mammalian tissues and is concerned with both receptors for hormones as well as receptors for a variety of other proteins such as immunoglobulins and serum proteins. In multicellular animals, communication amongst the cells is of prime importance and the receptors on cell surfaces represent the first line in communication amongst cells. Hence, the enormous interest in this area. In addition, by now techniques have become available to study in some detail both the chemical specificities of the receptors as well as the nature of the interaction of the receptors with communicating molecules.

Work continues in the Institute, in a tradition dating back to its inception, on the chemical nature of diseases caused by metabolic defects. This is the chemistry of genetic diseases. It is increasingly appreciated that a large number of human diseases are of genetic origin and hence the elucidation of their mechanism or etiology is of prime importance. This has continued apace in this Institute.

A common thread to many of these activities is interest in fundamental processes in biology. A change that appears gradually to be occurring, how-

ever, is a shift in interest of the most basic chemists, physicists, mathematicians, spectroscopists to the application of their tools and knowlege to problems that have a more direct bearing on problems of mankind. One gets the impression that there is beginning to be a sufficiency of framework in our knowledge of biological and biochemical processes so that it is now pertinent to investigate processes more closely related to those occurring in mammals or in man.

The continued scientific progress made by the Institute is, of course, totally dependent on its scientific staff. As opposed to a factory which produces some given product, a research institute is not as good as its equipment but only as good as its brains. In a factory that is well equipped when sufficient crew knowing how to operate the machines has been assembled and raw materials have been procured, the final product is almost inevitable. In a research institute, on the other hand, no matter how many machines there are, no matter how much raw materials are made available, and no matter how many workers are there to tend the machines which measure various parameters, there will be no product available unless there has been some ingenious concern to direct the use of the machines. Unfortunately there is no formula by which the experiments can be devised. New experiments, new ideas, new insights are only available through the unique genius of mankind.

Hence this Institute is not its buildings or equipment but its scientists.

ANNUAL REPORT SUMMARY

LABORATORY OF NUTRITION AND ENDOCRINOLOGY

It is with regret that we announce the retirement of Robert W. Bates after a long, productive and distinguished career in experimental endocrinology. His duties as Hormone Distribution Officer for NIAMDD have been accepted by Peter G. Condliffe.

During the past year, the Pilot Plant was finally moved from Building 3 to its new quarters attached to Building 6. By carefully controlling the scheduling and transfer of equipment, some operations were continued during the move. The new facility was in use in Building 6 seven weeks after the final shut-down in Building 3.

PILOT PLANT UNIT

Large-Scale Processing of Biological Materials

In spite of restricted operations a total of 226 requests were processed for investigators at the two locations: NIAMDD 90; NCI 63; NICHD 41; NHLI and NIAID 10 each; NINDS 3; NIDR, NIMH and BB 1 each; others 6. 118.5 kg quantities (60,360 liters of cultures) of 16 microorganisms were produced. The micro-organisms grown during this period included yeasts, molds and bacteria and consisted of wild type and mutant strains of *Arthobacter sp., Aspergillus oryzae, Bacillus amyloliquefaciens, Bacillus subtilis, Citrobacter sp., Comomonas sp., Chromobacter violaceum, Corynebacterium parvum, Escherichia coli, Haemophilus influenzae, Haemophilus parainfluenzae, Klebsiella cloaceae, Methanococcus vanneilii, Pneumococcus sp., Saccharomyces cerevisaie and Salmonella typhimurium.* Some of the organisms were used as hosts for the production of bacteriophage fd, MS2, Ø 80, lambda and T4, others were used to produce extracellular enzymes. Micro-organisms were grown 96 times in the 10 liter, 26 times in the 50-liter, 57 times in the 300-liter and 10 times in the 1100-liter fermentors. Of 189 fermentations, 24 (13%) were contaminated with 15 of these traced to the inocula supplied by the requesting investigators.

Studies were continued with the NICHD for polysaccharide production by *H. influenzae*, isolation of the enterotoxic proteins from special strains of *E. coli* and preparation of the protective antigen of typhoid fever from a non-pathogenic organism, *Citrobacter*. Spheroplasts from various strains of *E. coli* were produced five times for the NHLI using 10-, 50-, and 300-liter fermentors in series. The Gaulin laboratory homogenizer was used 42 times to rupture suspensions of yeast or bacterial cells. Large scale processing activities consisted of bovine milk xanthine oxidase from 20 gallons cream, separation of a three-enzyme mixture from rabbit muscle, the isolation of an inhibitor, barstar, from 7.4 kg *B. amyloliquefaciens*, and the preparation of two gallons of hydroxylapatite, a preparation preferred by several investigators over the commercial product for its superior resolving power and relatively fast flow rate. Various quantities of plant and animal tissue such as 200 lbs eel heads, 1 kg calf tendon and 5 lbs *Fagara xanthoides* roots were ground or sawed in the assorted laboratory mills and related equipment. Also, 16 kg pituitary glands and various aqueous solutions, such as ammonium acetate and carbonate, were lyophilized in the large Stokes freeze-dryer with a hot oil vacuum pump.

Culture fluids were concentrated in the Pfaudler and circulating-evaporator distilling devices.(D. L. Rogerson, Jr.)

GERM FREE RESEARCH UNIT

Studies in Experimental Nutrition.

Germfree, ex-germfree, and conventional animals are used to study the interrelationship between the host animal and its bacterial flora, and to identify the specific organisms involved. The effect of bacteria on the production of tumors is being studied, as well as the mechanism involved.

Experiments in which female rats are fed vitamin A acid or acetate result in the production and delivery of apparently healthy young which do not suckle because of the failure of the mothers ability to produce milk. This is an unusual nutritional problem and attempts are being made to correct this abnormality.

Possible interrelationships between vitamin A and the trace mineral, zinc, are being examined. Zinc appears to be necessary for the mobilization and transport of Vitamin A and its utilization at the actual functional site. There is preliminary evidence pointing to the involvement of zinc in the synthesis in the liver of retinol-binding-protein (RBP) which then mobilizes and transports the vitamin A as a retinol-RBP complex. Other relationships between vitamin A and zinc appear in Kwashiorkor, liver diseases and infections. Decreased blood levels of both vitamin A and zinc is an abnormality common to these three disease states. It is possible that the apparent vitamin A deficiency may be actually due to the insufficient amounts of zinc available in the blood. (J. Smith (V. A. Hospital) and E. G. McDaniel).

SECTION ON DEVELOPMENTAL BIOCHEMISTRY

Protein:Protein Interaction: Structure and Function of an Extracellular Ribonuclease (Barnase) and its Intracellular Inhibitor (Barstar).

Using both fluorescence of tryptophanyl residues and direct binding, an extensive study has been made of the phase transition induced in barnase by sodium dodecyl sulfate (SDS). Two theoretically independent methods of interpreting the fluorescence data suggest that native barnase does not bind SDS and that there is a two-state, direct transition to a transformed molecule which binds about 13 moles SDS/mole enzyme. Direct binding studies with [S^{35}] SDS confirm the lack of SDS binding to native barnase but suggest that larger amounts of detergent bind to the protein in the transition region, about 20 moles/mole.

A JEOLCO amino acid analyzer has been extensively modified to utilize the marked increase in detection sensitivity afforded by fluorescamine. Using conventional buffers, samples as small as 50 pmole are easily analyzed. Studies using different solvents and organic buffers for the analysis suggest that further simplification of the apparatus may be possible. (R. W. Hartley).

Structure and Function of Nucleic Acids.

S1 nuclease of *Asp. oryzae*, an enzyme specific for single-stranded regions of DNA or RNA, has been purified until free of any detectable contaminating

nuclease activities. The enzyme shows no base preference and hydrolyzes fd DNA in an apparently random fashion. In contrast, electrophoretically homogeneous, large oligonucleotides are obtained when S1 is used to hydrolyze MS2 RNA under defined conditions, suggesting that use of this nuclease should facilitate isolation of the base-paired regions of native RNA structures. Four oligomers, of size greater than tRNA, are present in such digests in large amounts and should be isolatable for sequence, biological and physical studies. (G. W. Rushizky).

Protein:Nucleic Acid Interaction: Chromatin Structure and Restriction in Eukaryotic Cells.

5-Deoxybromouridine (dBrU) selectively inhibits differentation in a number of established cell lines and embryonic organs. The mechanism of this inhibition of specialized macromolecule production is being investigated. HeLa cells, grown for one generation in dBrU and 5-deoxyfluorouridine have 85-90% of their DNA containing the thymidine analogue and hence banding at $\rho = 1.7500$ in CsCl. Although DNA synthesis is qualitatively the same in the presence of dBrU as in control cells, there appears to be a decreased rate of processing of 20-30 S DNA into high molecular weight nucleic acid, possibly related to a defect in replicon-replicon linkage. Chromatin containing dBrU has a composition similar to control chromatin and the histones and nonhistones of the two chromatins appear to be identical by gel electrophoresis. The inherent higher thermal stability of dBrU-containing DNA is also reflected in a 2-3° stabilization to thermal denaturation of dBrU chromatin. The most striking difference between the two chromatins is in DNA conformation - dBrU-chromatin having a circular dichroism spectrum totally unlike that of any normal chromatin. The possible relationships of this altered conformation to the molecular and cellular effects of the thymidine analogue are under investigation. (R. T. Simpson, R. L. Seale, and L. S. Propst).

Fractionation of Chromatin and the Study of Nonhistone Proteins

Further characterization of the histone and nonhistone chromatin proteins (NHCP) has been carried out on condensed and extended chromatin fractions from a variety of tissue sources. In all cases, extended chromatin is characterized by a diminution in its content of f1 histone. The NHCP of the two chromatin fractions show quantitative and occasional qualitative differences on SDS-polyacrylamide gel electrophoresis. There are not, however, any differences that are consistent for a given tissue of different species or for different tissues of a given species. (G. R. Reeck, R. T. Simpson, and H. A. Sober).

Duck erythrocyte chromatin, completely repressed *in vivo* and having a tissue-specific histone, f2c, was chromatographed on ECTHAM-cellulose. In contrast to all other chromatins, the usual fractionation into different molecular types was not observed. Thus, protein content and distribution, melting behavior and circular dichroism spectra were constant throughout the elution profile. Such results are consistent with this totally repressed tissue lacking any extended regions in its genome. (G. R. Reeck).

Small quantitative differences from normal parent tissue have been detected in the NHCP compositions of several transplantable rat hepatomas and renal tumors. Certain of these altered bands are present in greater or lesser than normal amounts in a number of tumor cell lines. (G. R. Reeck).

Metabolic Studies on Chromatin: Synthesis, Assembly and Turnover of Chromatin Constituents.

Chromatin assembly has been studied by examination of the nature of DNA synthesis during cycloheximide (CH) inhibition, which abolishes histone synthesis, as well as during processing of CH-DNA after removal of CH. The path of DNA synthesis in CH is like that of normal cells but 21S DNA fragments accumulate and are not processed to high molecular weight DNA while CH is present. After removal of the drug, these 21S DNA pieces are joined, but only over a time course of 3 hours as against 30 minutes in normal cells. The existence of small (less than 0.2% of the total chromatin proteins) pools of chromosomal proteins was demonstrated during CH inhibition of protein synthesis. The predominant protein pool was for f2a1, the highly conserved, arginine-rich histone. Although these pools exist, the majority of the chromatin DNA synthesized in CH is depleted in protein content, as indicated by its susceptibility to nuclease digestion. The degree of digestibility of CH-DNA is similar to that of briefly (less than 10 min) pulse-labeled control DNA, suggesting that comparable species occur in normal cells. The time course for the recovery of CH-DNA protein content after drug removal also parallels that for the processing of 21S fragments. These findings suggest that protein deposition on newly synthesized chromatin DNA occurs near the time of replicon-replicon linkage. (R. L. Seale and R. T. Simpson).

Attempts to isolate the messenger RNA for f1 histone are in progress. Polysomal RNA from mid-S phase HeLa cells is fractionated on a zonal sucrose gradient and the 5-15 S region is then subjected to preparative polyacrylamide gel electrophoresis. An 11.5 S fraction, suitable in size for f1 mRNA, has been isolated and shown to code for peptides with an appropriate amino acid composition for f1 histone when translated in a cell-free reticulocyte system. The mRNA still appears to be about 50% contaminated with other materials. (R. L. Seale).

Structural Analysis of the Active and Inactive Portions of the Eukaryotic Genome.

The nature of protein-DNA interactions in extended and condensed fractions of sonicated chromatin obtained by ECTHAM-cellulose chromatography have been explored. Condensed chromatin has less available DNA-phosphate (30%) than unfractionated chromatin both for digestion by micrococcal nuclease and for binding of polylysine. In contrast, the extended chromatin fractions have a higher content of available phosphate for both of these two probe molecules, *e.g.*, 70% of the DNA is digested by nuclease. Corroborative complementary data for binding of proteins has been obtained by acetylation of condensed and extended chromatin with acetic anhydride. Histones f2a1 and f2a2 do not appear to differ in their binding in the two fractions. In contrast, significantly more lysyl residues are modified in histones f2b and f3 in the extended chromatin fraction. Condensed chromatin apparently has a firm histone:DNA interaction while the extended chromatin has a loosened structure. (I. Polacow and R. T. Simpson).

The functional properties of these chromatin fractions, as exemplified by the frequency of binding sites for *E. coli* DNA-dependent RNA polymerase, were investigated by the method used by Cedar and Felsenfeld. Whole chromatin, with a polymerase binding site frequency 5-10% that of DNA, is resolved into condensed chromatin fractions with binding site frequencies of 2-4% that of DNA and extended chromatin fractions with binding site frequencies of 25-41%

that of DNA. Sub-fractionation of these chromatin fractions by sucrose gradient sedimentation leads to a slight further purification of repressed and transcribable segments of the genome. Overall, it is possible to obtain fractions of a given chromatin which differ by 25 to 50-fold in their frequency of polymerase binding sites. (R. T. Simpson and L. S. Propst).

Biochemistry of Pituitary Glycoprotein Hormones.

Purified bovine TSH and LH are being prepared from frozen pituitaries. The purification of TSH is approached through development of an affinity column method. IgG antibody fractions from the serum of a burro immunized with bovine TSH are coupled to activated Sepharose. Investigation of possible prohormones and the role of proteases in the generation of active pituitary hormones will be expedited by development of this fractionation method. (P. G. Condliffe and N. Yulug).

SECTION ON ENDOCRINOLOGY

Hormonal Induction of Diabetes.

Hormonal induction of diabetes in rats has been studied in this laboratory for nearly 10 years. Although rats are notable for their resistance to hormonally induced diabetes, severe diabetes could be developed in partially (60-80%) pancreatectomized rats when implanted with pituitary tumors (MtT-F4) that secreted large amounts of prolactin, growth hormone and ACTH. Subsequent studies in partially pancreatectomized and intact rats showed that the administration of large amounts of growth hormone, ACTH and adrenal glucocorticoids produced diabetes, and that the diabetogenic effects of growth hormone and glucocorticoids were synergistic. Tolbutamide was also found to be diabetogenic in that it decreased both the amount of hormone needed to induce glucosuria and the concentration of insulin in the pancreas.

Partially pancreatectomized rats implanted with MtT-F4 tumor were used to observe changes in concentration of glucose in blood and of insulin in serum and pancreas during the development of diabetes. However, individual differences in rates of growth, onset of glucosuria, etc., were such that the sequence of events could not be determined. Therefore, the study was repeated in rats made diabetic by injection of hormones. Intact rats, given frequent injections of diabetogenic hormones, showed the following changes: Serum insulin concentration increased several fold during the first few hours and remained high, pancreatic insulin concentration was normal on the first day but then decreased 75% during the next 3-4 days, and hyperglycemia and glucosuria developed on or after the third day of treatment. When injections were continued beyond 5 days, the pancreatic insulin concentration returned to normal (2 IU/g), serum insulin concentration remained high, and hyperglycemia and glucosuria persisted. After hormonal injections were stopped, blood glucose returned to normal in 1-2 days, glucosuria disappeared, serum insulin concentration decreased, and pancreatic insulin concentration increased for a short time. These studies indicated that pancreatic beta cells in the intact rat are able to produce large amounts of insulin in response to diabetogenic hormones, thus maintaining the serum insulin at a high level.

Since it was not practicable to inject large amounts of hormones for more than 2 weeks, intact rats bearing MtT-F4 tumors were used to study the effect

of prolonged hormonal stimulation (growth hormone and ACTH) on insulin resistance. Serum and pancreatic insulin concentrations were both increased 3-to-4-fold above normal in rats studied 1-2 months after tumors became palpable, *i.e.*, secretory. Hyperglycemia and glucosuria, however, did not develop in tumor bearing rats unless part of the pancreas had been removed. When 80% of the gland was removed, insulin concentration in the remaining pancreatic tissue fell below 10% of normal, serum insulin concentration also dropped, and severe, often permanent, diabetes ensued. Collapse of the pancreatic beta cells in these animals was confirmed by histological evidence of cellular degranulation and glycogen accumulation. (R. W. Bates).

Effect of Hormones on Lipid Mobilization.

Two independent processes are involved in mobilization of fatty acids from adipose tissue -- lipolysis and fatty acid re-esterification. The former is a hormone-activated process, usually involving cyclic-AMP. The latter is dependent on the formation of α-glycerophosphate from glucose. The effect of ACTH and adrenalectomy on these processes was studied in rat adipose tissue perfused *in vivo*. Injection of ACTH in normal animals increased lipolysis but not re-esterification and resulted in increased mobilization of fatty acids from adipose tissue. Adrenalectomy, on the other hand, decreased lipolysis and reduced release of fatty acids. ACTH injection in adrenalectomized rats, however, increased both lipolysis and fatty acid re-esterification and thus produced very little change in fatty acid release from the tissue. It is concluded that adipose tissue in adrenalectomized rats is sensitive to the lipolytic action of ACTH, but increased efficiency of glucose utilization in the absence of glucocorticoid secretion enhances re-esterification of fatty acids in fat cells. This probably explains the apparent ineffectiveness of ACTH in fatty acid mobilization in adrenalectomized animals. (S. S. Chernick and V. Kovacev (Univ. of Skopje, Skopje, Yugoslavia)).

Pharyngeal (Lingual) Lipase.

Early studies in this laboratory showed that serous glands in the tongue and pharyngeal tissues of the rat secrete a very potent enzyme which hydrolyzes triglyceride primarily to diglyceride and free fatty acid (FFA) at pH 4.5-5.5. This enzyme accounts for most of the lipolytic activity found in the stomach. More recent studies showed that the enzyme does not need an acceptor for the FFA produced which can accumulate in the oily substrate. Addition of albumin, however, but not Ca^{++}, enhances activity of the enzyme, producing complete hydrolysis of at least 70% of the substrate to FFA and glycerol.

Recent studies in human volunteers have shown a similar lipolytic activity in aspirates collected from the upper and lower esophagus and stomach. Secretions obtained from the major salivary glands did not contain lipolytic activity, thus excluding these tissues as sources of the enzyme. The findings indicate that pharyngeal tissue in man secrete an enzyme, pharyngeal lipase, which acts in the stomach to hydrolyze dietary triglyceride to partial glyceride and FFA. It is proposed that this is the first step in the digestion of dietary fat in man. (M. Hamosh, R. O. Scow, H. L. Klaeveman (A:D&HD) and R. O. Wolf (D:OMS)).

Metabolism of Chylomicrons by Lactating Rat Mammary Gland .

Perfused mammary glands of lactating rats take up glyceride-fatty acids and glycerol and cholesterol from circulating chylomicrons. Hypophysectomy, one day before perfusion, markedly lowered lipoprotein lipase activity and tissue uptake of both glyceride and cholesterol. Radioautographic studies showed that fatty acids and glycerol taken up were readily incorporated into milk lipid. Other studies are now in progress to determine the fate of cholesterol removed from chylomicrons. Earlier results showed that mammary gland lipoprotein lipase activity is regulated by the anterior pituitary gland through the secretion of prolactin. (E. J. Blanchette-Mackie, C. R. Mendelson, O. Zinder and R. O. Scow).

Cytochemical studies in the electron microscope showed that lipoprotein lipase activity is present in the endothelial cells of capillaries in lactating mammary gland. The findings also indicate that glyceride derived from chylomicrons is transported across the capillary endothelium in microvesicles and vacuoles. (E. J. Blanchette-Mackie, G. Devore and R. O. Scow).

Effect of Lipoprotein Lipase on Chylomicrons *in vitro* .

Purified lipoprotein lipase, obtained from bovine milk, readily hydrolyzes chylomicron triglyceride to FFA and glycerol if sufficient albumin is present in the medium to bind the FFA produced. Otherwise, hydrolysis is limited and FFA accumulates inside the chylomicron. However, if albumin is present in great excess, 4-8 times that needed to bind FFA (6 mol/mol albumin), two-thirds of the monoglyceride formed during hydrolysis is trapped outside of the chylomicron and hydrolysis is incomplete. Lipoprotein lipase also hydrolyzes lecithin in the surface film of chylomicrons to 2-acyl lysolecithin, and a large proportion of the lysolecithin remains without disrupting the chylomicron. Incubation of chylomicrons with phospholipase-C, which removes the phosphorylcholine moiety from lecithin,converts lecithin in the surface film to diglyceride without affecting either the stability of the lipid particles, or the action of lipoprotein lipase on core triglyceride. These findings indicate that the polar groups of lecithin, which constitutes 4/5 of the surface film, are not necessary for the interaction of lipoprotein lipase and chylomicrons.

Electron microscopic studies reported earlier showed that the chylomicrons are enveloped by a monolayer surface film. The surface film could be visualized only after the chylomicron core had been reduced by action of lipoprotein lipase from postheparin plasma on core triglyceride. The studies have been extended using purified lipoprotein lipase from milk to determine the early changes induced in chylomicron structure. It was found that FFA, and also water, accumulate within the chylomicron if the incubation medium does not contain sufficient albumin to accept the FFA produced. Retention of FFA and water in chylomicrons was verified by scanning electron microscopy. The morphological appearance of lipolyzed chylomicrons was markedly affected by pH during the preparation of the specimens. Studies with the freeze-etching technique indicate that the surface film of chylomicrons changes when exposed to lipoprotein lipase. Examination of the relationship between these changes and the presence of the enzyme are now being made. (E. J. Blanchette-Mackie and R. O. Scow).

Role of Guanine Nucleotides in Activity and Hormonal Regulation in Adenylate Cyclase Systems.

Previous studies have shown that GTP and glucagon activate the glucagon-sensitive adenylate cyclase system in hepatic plasma membranes by a concerted or interdependent process. Kinetic studies carried out with limiting concentrations of substrate (MgATP) have revealed the following: glucagon, by a co-operative binding process, facilitates the reactivity of GTP with a regulatory process involved in activation of adenylate cyclase. GTP^{-4} is the activating form of the nucleotide; the minimal effective concentration of free GTP is about 10^{-10} M. ATP, at concentrations 3-4 orders of magnitude higher than GTP, may substitute for GTP. However, it is possible that the effects of ATP are due to contaminating GTP present in commercial preparations of ATP. Activation of the enzyme by GTP does not involve significant changes in the K_m (10^{-5} M); V_{max} is increased 4-fold. The GTP-activated state displays a decreased affinity for $HATP^{-3}$, a potent inhibitor ($K_i = 10^{-8}$ M) of the enzyme, (presumably at the catalytic site).

Gpp(NH)p, an analogue of GTP that is not hydrolyzed by nucleotide phosphohydrolases in plasma membranes, also activates the hepatic system synergistically with glucagon. However, in contrast to GTP, Gpp(NH)p causes nearly full activation in the absence of glucagon. Gpp(NH)p exhibits lags in onset of activation while glucagon abolishes the lag and appears to increase the rate at which Gpp(NH)p activates the enzyme. Activation by Gpp(NH)p alone is very temperature-dependent; temperatures above 37° convert the enzyme to a fully activated state that is only slowly reversed to basal state even after washing the membranes. Labeled Gpp(NH)p binds tightly to membranes. Pretreatment of membranes with labeled Gpp(NH)p followed by washing, yields levels of adenylate cyclase activity commensurate with the amount of Gpp(NH)p remaining bound. GTP or GDP displace Gpp(NH)p from membranes, thus inhibiting activation. However, GTP requires glucagon for activation of the enzyme system whereas GDP does not activate the enzyme in the absence or presence of glucagon.

The ACTH-sensitive adenylate cyclase system in rat adrenal cells displays essentially the same overall kinetic characteristics of the hepatic system. We have failed to confirm findings of others that calcium ion is involved. Gpp(NH)p acts synergistically with ACTH and, as in the case of the hepatic system, also causes activation in the absence of ACTH by a time- and temperature-dependent action.

Two strains of mouse adrenal tumor cell lines, one (Y-1) containing an ACTH-sensitive and another (OS3) with an ACTH-insensitive adenylate cyclase system, are both activated by Gpp(NH)p. However, the OS3 adenylate cyclase requires higher temperatures for activation than does the Y-1 system. The basis of the different temperature dependencies remains unknown. It is important to note, however, that Gpp(NH)p activates adenylate cyclase even in the absence of a functional hormone receptor.

Gpp(NH)p activates adenylate cyclase systems in all eukaryotic cells investigated. For example, Gpp(NH)p activates the multi-receptor fat cell system, (sensitive to glucagon, secretin, ACTH, epinephrine, TSH), the catecholamine-sensitive systems in avian, amphibian, and fish erythrocytes, the prostaglandin-sensitive systems in human platelets and bovine thyroid, and the TSH-sensitive systems in thyroid. All of these display the same characteristics with respect

to lag in onset of activation by the nucleotide, temperature requirements, and synergism with hormones. The ubiquitous effect of GPP(NH)p suggests a common mechanism in eukaryotic cells involving a guanine nucleotide-dependent process. In the one bacterial system investigated, that in *E. coli* B, Gpp(NH)p did not alter enzyme activity.

The fat cell system has provided the strongest evidence that adenylate cyclase systems are composed of subunits linked to form a cooperatively responding enzyme system, activatable by several hormones, each of which binds to distinct receptors. Each hormone requires GTP (or Gpp(NH)p) for activation. With minimal nucleotide concentrations, hormones in various combinations give additive responses. With GTP or Gpp(NH)p, addition of secretin, the most active hormone, results in larger activity increases than can be accounted for by a non-cooperating system. However, increasing concentrations of nucleotide abolish the additive effect. These findings suggest that the fat cell adenylate cyclase system is composed of heterologous subunits each of which is coupled to distinct receptors for each hormone. Subunit interaction and expression of adenylate cyclase activity in response to hormones appear to be altered by the binding of guanine nucleotides to the nucleotide regulatory sites. (M. Rodbell, M. C. Lin, Y. Salomon, C. Londos, M. Schramm and B. R. Martin).

SECTION ON NUTRITIONAL BIOCHEMISTRY

Metabolism of Fat-Soluble Vitamins.

Studies designed to evaluate the vitamin E adequacy of corn, soybean and safflower oils were conducted by feeding these oils as 20% of the diet. Several biochemical and histological criteria indicated that the vitamin E status of the animals was satisfactory. Analyses permitted the calculation of molar ratios of polyunsaturated fatty acids:α-tocopherol for six tissues. These ratios are proposed as indices of tissue vitamin E adequacy (J. G. Bieri and R. Poukka-Evarts).

Net Uptake and Metabolism of Plasma Glutamine by Intestine.

The rat small intestine has been found to be unique among tissues in its utilization of large amounts of glutamine, sufficient to cause a turnover of the entire plasma glutamine pool in five minutes. Of the total CO_2 production by the intestine, 32% came from glutamine. The nitrogen of metabolized glutamine was found in citrulline, alanine, and ammonia (23-33%) and in proline (10%). The mucosa was characterized as the most active portion of the intestine in metabolizing glutamine. (H. G. Windmueller).

Biochemical Studies of Cytochromes.

Analyses of mitochondrial and submitochondrial liver fractions by linear gradient distribution have revealed that lysosomes and peroxisomes are not homogeneous but each has two distinct sub-populations. Six sub-fractions of purified mitochondria were obtained and characterized by their enzyme activities. It was concluded that some of these mitochondria have outer membranes that are still contiguous with the endoplasmic reticulum. Studies of the binding of cytochrome c to outer and innner mitochondrial membranes indicate that the outer membrane has many more binding sites than the inner membrane. (J. N. Williams).

SECTION ON VITAMIN METABOLISM

Dihydrofolic Acid Reducatase.

This enzyme which catalyzes the reduction of folic or dihydrofolic acid to the metabolically active form, tetrahydrofolate, is the target for the important anti-cancer drug, methotrexate. Since its discovery in this laboratory in 1954, it has been difficult to purify because of its lability and the minute amounts present in animal tissues. Very significant progress has been made by the development of an affinity chromatographic system consisting of an adsorbent prepared by coupling methotrexate to agarose via a six carbon arm. In general, the procedure used for the purification of dihydrofolic reductase from beef liver is identical to that previously reported for the chicken liver enzyme. However, the enzyme preparation after elution appears to contain high molecular weight impurities which require an additional gel-filtration for their removal. Further examination of the highly purified enzyme revealed that it contains tightly bound dihydrofolate far in excess of that expected from kinetic data. Conventional treatment with DEAE-cellulose and hydroxylapatite was only partially successful in the removal of the bound substrates. However a completely "clean" enzyme preparation could be prepared by a combination of isoelectric focusing and hydroxylapatite chromatography. Reexamination of the chicken liver enzyme also revealed the presence of tightly bound substrates. These could also be removed by the combination of isoelectric focusing and hydroxylapatite chromatography. (B. T. Kaufman).

Properties of Folic Acid γ-Glutamyl Carboxypeptidase.

Studies on the mechanism of hydrolysis of yeast and *Pediococcus cerevisiae* folates by conjugases from chicken liver and pancreas have been made using DEAE-cellulose thin-layer chromatography. Diglutamates and monoglutamates are produced by the two enzymes, respectively. Liver conjugase apparently removes glutamyl residues in a stepwise manner, one at a time, from *Pediococcus* folate. A mild procedure has been developed for purification of folates from any source and applied to *Pediococcus*. After disruption of cells with sodium dodecyl sulfate and sonication, centrifugation and gel filtration allow preparation of material which can be directly fractionated on DEAE-cellulose. (G. R. Reeck and H. Bakerman).

COLLABORATIVE RESEARCH

Effects of Hormone Metabolism of Adipose Tissue Perfused *in vivo*:-
With Dr. Vance Kovacev, Department of Physiology, Medical Faculty, University of Skopje, Skopje, Macedonia, Yugoslavia (S. S. Chernick).

Ultrastructural Aspects of Lipid Metabolism:-
with Dr. Olga Stein, Department of Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem Israel (R. O. Scow).

Control of Lipid Synthesis by the Liver:-
with Dr. Benyamin Shapiro, Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel (M. Rodbell).

Metabolic Studies of Vitamin A:-
with Dr. J. Ganguly, Department of Biochemistry, Indian Institute of Science, Bangalore, India (J. Bieri).

Effect of DNA and DNA Nucleotides in the Reaction of the Immune Response:-
with Dr. Michael Feldman, Department of Cell Biology, Weizmann Institute of Science, Rehovoth, Israel (H. A. Sober).

A Study of Individual Oligopeptide Members of Polyamino Acid Series:-
with Dr. Ephraim Katchalski, Department of Biophysics, Weizmann Insitute of Science, Rehovoth, Israel (H. A. Sober).

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Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on folic acid.

Previous Serial Number: SAME

Principal Investigator: Dr. B. T. Kaufman

Other Investigators: Ms. R. C. Gardiner and Ms. M. K. Romine

Cooperating Units: None

Man Years

Total:	3.0
Professional:	1.0
Other:	2.0

Project Description:

Objectives: To isolate and determine the biochemical properties of enzymes concerned with the metabolism of folic acid, especially dihydrofolic reductase from chicken liver, beef liver, yeast, bacteria and certain leukemic tumor cells. To study the interaction of these enzymes with agents known to produce activation, as well as with certain anti-leukemic drugs which result in inactivation. To isolate and determine the chemical and biological properties of the naturally occurring forms of folic acid in various cells and tissues.

Methods Employed: Standard chemical and biochemical techniques appropriate for the purification and fractionation of proteins and vitamins are used in these projects in conjunction with various methods of chemical, physical, enzymatic and microbiological analysis.

Major Findings: The previously reported successful purification of chicken liver dihydrofolic reductase via affinity chromatography using a column of methotrexate coupled to Sepharose has been extended to the large scale purification of the enzyme from calf liver. Initial examinations of calf liver dihydrofolic reductase revealed that although many of the properties of the calf liver enzyme were quite similar to those of the chicken liver enzyme, other properties were quite different. Despite these differences, procedures developed for the chicken liver enzyme prior to the affinity column adsorption were found to be applicable to a calf liver preparation. Thus, calf liver could be homogenized in dilute $MnCl_2$, yielding a clear extract, which in turn, could be further purified by treatment with zinc hydroxide gel. As in the case of the chicken liver enzyme, the calf dihydrofolic reductase is temporarily

inactivated by this procedure. However, full recovery of activity is obtained by treatment with EDTA and precipitation of the protein with ammonium sulfate. The resuspended precipitate can now be directly adsorbed onto the affinity column.

Using these procedures, approximately 80-90% of the original dihydrofolic reductase activity has been adsorbed from 10 kilos of calf liver by adding the extract, 600 g at a time, to the methotrexate-Sepharose column (25 x 100 mm) over a period of 20 days. Elution of the column yielded about 50 mg of enzyme representing approximately 60% recovery. Subsequent passage of the enzyme through a Sephadex G-75 column (20 x 1000 mm) yielded a preparation which appeared to be homogeneous despite the observation that additional absorption and elution from DEAE-cellulose and hydroxylapatite gave further increases in specific activity. Investigation of this phenomenon revealed that the enzyme from the gel filtration step contained large amounts of very tightly bound substrate, dihydrofolate, which, doubtlessly had been derived from the substrate used for the elution of the affinity column. Treatment with both DEAE-cellulose and hydroxylapatite were partially successful in removing this material.

Further studies with the "pure" enzymes eluted from the methotrexate-column revealed that both the chicken and beef enzyme contained extremely tightly bound dihydrofolate far in excess of what would be expected from kinetic data. It has been virtually impossible to remove this substrate by conventional techniques. However, it was discovered that completely "clean" enzyme could be prepared by isoelectric focusing. This procedure not only removes tightly bound substrates but can also remove methotrexate from the enzymes which has heretofore not been possible.

Electrofocusing has revealed multiple peaks of activity as well as some unusual differences between the chicken and beef enzymes. Preliminary studies on the activation phenomenon with the beef liver enzyme revealed that although high concentrations of urea and thiourea resulted in stimulation of enzyme activity in a manner similar to that observed with the chicken liver enzyme, stoichiometric activating agents such as mercurials, iodine and tetrathionate produced only inhibition.

Other investigators describing the purification of beef liver dihydrofolic reductase by means of conventional enzyme purification procedures, have reported that the final product exhibited a specific activity of 1 μmole of dihydrofolate reduced per min per mg of protein. Our enzyme preparation obtained from calf liver by affinity chromatography exhibited a specific activity of 20 as did our preparation from beef liver.

Significance to Bio-Medical Research and the Program of the Institute:
Dihydrofolic reductase is not only a key enzyme in the metabolism of folic acid, it is also of major importance in the synthesis of thymidine and the chief site of action of the anti-folic drugs. Thus, the understanding of the structure and function of this enzyme would be of basic importance in both normal metabolism and in cancer chemotherapy. Folic acid, as such, most probably does not occur in nature. Thus, the knowledge of chemical structure and biological properties of the more complex natural form will result in a better understanding of the biochemistry of this vitamin.

Proposed Course of Project: Now that for the first time reasonably large amounts of completely pure enzyme from both beef and chicken are available, investigations can begin to examine the physical properties and mechanism of of these two liver dihydrofolic reductases with particular emphasis on the nature of the activations and inhibitions.

Honors and Awards: NONE

Publicatons: NONE

Serial No. NIAMDD-LNE-2
1. Nutrition and Endocrinology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Large-scale processing of biological materials.

Previous Serial Number: SAME

Principal Investigator: Mr. D. L. Rogerson, Jr.

Other Investigators: Dr. H. A. Sober

Cooperating Units: None

Man Years

Total:	3.5
Professional:	1.0
Other:	2.5

Objectives: To provide facilities and services for the processing and/or production of large amounts of natural materials, such as bacteria, animal tissues, cells, blood, urine and plant materials, so that they may be studied under standard laboratory conditions.

Methods Employed: To satisfy the large-scale production needs of NIH investigators, methods are designed for the scaling up of small laboratory experiments utilizing large reaction vessels, extractors, stills, concentrators, centrifuges, etc., so that the processing can be carried out effectively, efficiently and safely. Two 10-liter, one 50-liter, two 300-liter and one 1100-liter fermentors are utilized for growing microorganisms under a variety of conditions. Bacterial cells and yeasts are harvested by means of laboratory or large-scale AS-16P and AS-26P centrifuges. Protozoa are concentrated in continuous flow centrifuges. Cells are either supplied as a paste or further processed to yield cell-free suspensions by rupture with a Gaulin homogenizer. In some instances, a specific substance is isolated and partially purified by further processing of the culture supernatant or the cell paste using conventional fractionation procedures and the newer chromatographic techniques.

Major Findings: During the past year, the fermentors, centrifuges and other equipment of the Pilot Plant were moved from Building 3 into a new addition to Building 6. By carefully controlling the transfer of individual pieces of equipment, we were able to continue some operations in Building 3, during the move. Limited operations were resumed in Building 6 seven weeks after the final shut-down in Building 3. Despite restricted operations, a total of 226 requests were processed for investigators at the two locations: NIAMDD 90; NCI 63; NICHD 41; NHLI and NIAID 10 each; NINDS 3; NIDR, NIMH and

BB 1 each; others 6. 118.5 kg quantities (60,360 liters of cultures) of 16 microorganisms were produced. The microorganisms grown during this period included yeasts, molds and bacteria and consisted of wild type and mutant strains of *Arthobacter sp., Aspergillus oryzae, Bacillus amyloliquefaciens, Bacillus subtilis, Citrobacter sp., Comomonas sp., Chromobacter violaceum, Corynebacterium parvum, Escherichia coli, Haemophilus influenzae, Haemophilus parainfluenzae, Klebsiella cloaceae, Methanococcus vanneilii, Pneumococcus sp., Saccharomyces cerevisaie and Salmonella typhimurium.* Some of the organisms were used as hosts for the production of bacteriophage fd, MS2, Ø 80, lambda and T4, others were used to produce extracellular enzymes. Micro-organisms were grown 96 times in the 10 liter, 26 times in the 50-liter, 57 times in the 300-liter and 10 times in the 1100-liter fermentors. Of 189 fermentations, 24 (13%) were contaminated with 15 of these traced to the inocula supplied by the requesting investigators.

Studies were continued with the NICHD for polysaccharide production by *H. influenzae,* isolation of the enterotoxic proteins from special strains of *E. coli* and preparation of the protective antigen of typhoid fever from a non-pathogenic organism, *Citrobacter.* Spheroplasts from various strains of *E. coli* were produced five times for the NHLI using 10-, 50-, and 300-liter fermentors in series. The Gaulin laboratory homogenizer was used 42 times to rupture suspensions of yeast or bacterial cells. Large scale processing activities consisted of bovine milk xanthine oxidase from 20 gallons cream, separation of a three-enzyme mixture from rabbit muscle, the isolation of an inhibitor, barstar, from 7.4 kg *B. amyloliquefaciens,* and the preparation of two gallons of hydroxylapatite, a preparation preferred by several investigators over the commercial product for its superior resolving power and relatively fast flow rate. Various quantities of plant and animal tissue such as 200 lbs eel heads, 1 kg calf tendon and 5 lbs *Fagara xanthoides* roots were ground or sawed in the assorted laboratory mills and related equipment. Also, 16 kg pituitary glands and various aqueous solutions, such as ammonium acetate and carbonate, were lyophilized in the large Stokes freeze-dryer with a hot oil vacuum pump. Culture fluids were concentrated in the Pfaudler and circulating-evaporator distilling devices.

Significance to Bio-Medical Research and the Program of the Institute: Within the laboratory's facilties many projects can be assisted and facilitated whenever large amounts of material have to be produced or processed.

Proposed Course of Project: To continue assisting NIH investigators when large-scale processing of biological materials is needed in their projects and to expand the facilities and capabilities for the isolation and purification of biologically-important macromolecules, cell particulates and materials.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAMDD-LNE-3
1. Nutrition and Endocrinology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
INDIVIDUAL PROJECT REPORT
July 1, 1973 through June 30, 1974

Project Title: Studies in experimental nutrition.

Previous Serial Number: Same

Principal Investigator: Mr. E. G. McDaniel

Other Investigators: Dr. F. S. Doft, Guest Worker, (LNE,NIAMDD)
Dr. G. Laqueur (LEP, NIAMDD)
Dr. J. Smith (VA Hospital, Trace Element Lab)

Cooperating Units: None

Man Years

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Objectives: To study the nutritional, biochemical and physiological characteristics of germfree, ex-germfree and conventional animals and the influence of bacterial flora upon these characteristics.

Methods Employed: Animals are maintained on purified diets of known composition in which individual dietary factors may be controlled. Growth, blood and tissue values as well as other physiological functions are evaluated. Previously germfree animals may be inoculated with strains of bacteria to identify the organisms responsible for various phenomena. Germfree and conventional animals are used as controls.

Major Findings: With Dr. Laqueur, studies of the action of cycasin have shown the compound to be non-toxic to adult germfree rats. It does produce tumors in conventional rats, in ex-germfree rats infected with certain bacteria, and in very young, germfree rats. However, the aglycone of cycasin produces tumors in both germfree and conventional rats.

Tumors occur in animals infected with specific strains of bacteria known to convert cycasin to its aglycone as in conventional animals. These observations indicate that, except for the very young rat, it is necessary for bacteria to convert cycasin to its aglycone for its toxic effects to be observed. It appears that the young rat does possess within its tissues

enzymes capable of making such a conversion, but this capability is lost rather early in life.

It is difficult to produce simultaneous deficiencies of both vitamin A and zinc in the young rat, since zinc deficiency occurs quite rapidly but vitamin A deficiency requires several weeks. The much slower onset of vitamin A deficiency is probably due to the stores of the vitamin whereas zinc is not stored to any appreciable degree. We have attempted to avoid this discrepancy by using young rats from mothers maintained on vitamin A-acid which is not stored in the body. However, an unexpected finding was that the females delivered apparently healthy babies but were unable to nurse them. It was shown that the difficulty was also observed when Vitamin A acetate was used. The young rats appear normal with respect to numbers, size and activity and will survive up to seven days even though no milk is consumed. Milk was found in the stomachs of foster-nursed young rats, indicating that the abnormality is an inability of the mother to produce milk, rather than some deficiency in the young. Although these diets are semi-purified, they are of known composition and considered to be adequate.

With Dr. Smith, experiments to study the development, prevention or treatment of vitamin A and of zinc deficiencies have been continued. It has been shown that zinc deficient rats have lower than normal levels of plasma vitamin A, even though adequate A is supplied and the liver contains normal levels of the vitamin. With a deficiency of both vitamin A and zinc, the liver vitamin A also becomes abnormally low. When the doubly-deficient animal is treated with vitamin A alone, the liver A is elevated but the plasma level remains low. Administration of zinc alone produces an increase in plasma vitamin A even though liver stores are very low. Treatment with both factors results in an increase in both liver and plasma. These observations suggest that zinc is necessary to mobilize vitamin A from liver storage sites.

Zinc deficiency in the rat was accompanied by a marked decrease in the levels of retinol-binding protein (RBP) in both plasma and liver. It is, therefore, suggested that zinc may also be essential for the synthesis of RBP in the rat tissue.

Humans with Kwashiorkor have decreased levels of vitamin A and zinc in the blood. We are attempting to produce a somewhat similar situation in the rat by feeding inadequate levels of protein. We have observed that in germ-free rats fed diets containing 3% casein, plasma vitamin A levels become decreased, even though animals are given adequate amounts of vitamin A orally and zinc intraperitoneally. It was further observed that these rats developed a dermatitis on the paws similar to that seen during zinc deficiency.

It is known that the sense of taste is impaired during periods of deficiency of zinc or vitamin A. Experiments are being done to determine if this effect can be used to measure the progress of development or treatment of a deficiency in the rat, and to see if the effect on taste observed during zinc deficiency may actually be due to an inability to utilize stored vitamin A.

Significance to Bio-Medical Research and the Program of the Institute: The use of germfree, ex-germfree and conventional animals makes it possible to study both the beneficial and deleterious ways in which an animal is influenced by its intestinal flora. In nutritional studies, germfree animals can be maintained in a much more severe state of dietary deficiency than conventional animals. The germfree animal, therefore, provides a convenient biological tool for the examination of the abnormalities of nutritional or other diseases in the absence of complications accompanying the presence of bacteria.

Proposed Course of Project: Experiments will be continued to study the relationship between the host animal and its bacterial flora with respect to its influence upon the nutrition and physiology of the host. Identification of specific organisms responsible for such influence will be attempted. Efforts will be continued to develop techniques by which the germfree animal may serve as an experimental tool in conjunction with clinical research.

Honors and Awards: NONE

Publications:

Smith, J. C., McDaniel, E. G., and Doft, F. S.: Urinary calculi in germfree rats: Alleviated by varying the dietary minerals. In Heneghan, J. B. (ed): Germfree Research: Biological Effect of Gnotobiotic Environments. New York, N. Y., Academic Press, 1973, pp. 285-290.

Smith, J. C., McDaniel, E. G., Fan, F. F., and Halsted, J. A.: Zinc: A trace element essential in vitamin A metabolism. Science 181: 954-955, 1973.

Serial No. NIAMDD-LNE-4
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Protein Hormones: Chemistry and Physiological Actions of Protein Hormones

Previous Serial Number: SAME

Principal Investigator: Dr. Robert W. Bates

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1-1/2
Professional:	1/2
Other:	1

Project Description:

Objectives: To isolate protein hormones from pituitary glands of man and animals, functional transplantable mouse and rat pituitary tumors and blood; to investigate the nature of these hormones as they circulate in the blood stream; to study their effects when produced in excess by tumors; to investigate the induction of diabetes by hormones.

Methods Employed: Hormones and other substances are extracted from pituitary gland, rat and mouse pituitary tumors, pancreas and blood. Crude extracts which may contain several hormones are fractionated by techniques commonly used in protein chemistry, such as salt precipitation, solvent fractionation, isoelectric precipitation, preparative and disc electrophoresis, ion-exchange chromatography, and gel filtration. Various quantitative biological assays are required to determine the concentration of the several hormones in tissues and protein fractions. Radioimmunoassay techniques are employed to extend the studies to concentration levels below the reach of conventional bioassay procedures. Removal of various endocrine glands with and without hormonal replacement is a critical technique. Measure glucose in blood and urine, and insulin in blood and pancreas of intact and partially pancreatectomized rats, either bearing transplantable pituitary tumors or given different combinations of hormones and other substances.

Major Findings: Hormonal Induction of Diabetes: Hormonal induction of diabetes in rats has been studied in this laboratory for nearly 10 years. Although rats are notable for their resistance to hormonally induced diabetes, severe diabetes developed in partially (60-80%) pancreatectomized rats when

implanted with pituitary tumors (MtT-F4) that secreted large amounts of prolactin, growth hormone and ACTH. Subsequent studies of the action of these and other hormones in partially (60-80%)pancreatectomized and intact rats showed that growth hormone, ACTH and adrenal glucocorticoids given in large amounts produced diabetes, and that the diabetogenic effects of growth hormone and glucocorticoids were synergistic. Tolbutamide was also found to be diabetogenic in that it decreased both the amounts of hormones needed to induce glucosuria and the concentration of insulin in the pancreas.

Rats that had been partially pancreatectomized and implanted with MtT-F4 tumor were used to determine changes in concentration of glucose in blood and of insulin in serum and pancreas during the development of diabetes. However, differences between rats, in rates of tumor growth, onset of glucosuria, etc., were so large it was not possible to draw any conclusion concerning the sequence of events. Therefore, the study was repeated in rats made diabetic by injection of hormones.

Intact rats given frequent injections of diabetogenic hormones showed the following changes: Serum insulin concentration increased several fold during the first few hours and remained high, pancreatic insulin concentration was normal on the first day and then decreased 75% during the next 3-4 days, and hyperglycemia and glucosuria developed on or after the third day of treatment. When injections were continued beyond 5 days, the insulin concentration in pancreas increased to normal (2 IU/g), serum insulin concentration remained high, and hyperglycemia and glucosuria persisted. When hormonal injections were stopped, blood glucose returned to normal in 1-2 days, glucosuria disappeared, serum insulin concentration decreased, and pancreatic insulin concentration increased for a short time. These studies indicated that pancreatic beta cells in the intact rat are able to produce large amounts of insulin in response to diabetogenic hormones and thus maintain the concentration of insulin in serum at a high level.

Since it was not practicable to inject large amounts of hormones for more than 2 weeks, intact rats bearing MtT-F4 tumors were used to study the effect of prolonged hormonal stimulation (growth hormone and ACTH) on insulin resistance. Serum and pancreatic insulin concentrations were both increased 3-4 times above normal in rats studied 1-2 months after tumors became palpable, i.e., secretory. Hyperglycemia and glucosuria, however, did not develop in tumor bearing rats unless part of the pancreas had been removed. When 80% of the gland was removed, insulin concentration in the remaining pancreatic tissue fell below 10% of normal, serum insulin concentration also dropped, and severe, often permanent, diabetes ensued. Collapse of the pancreatic beta cells in these animals was confirmed by histological evidence of degranulation and glycogen accumulation in the cells.

Effect of hormones on organ weights: Organ weight data were obtained from rats used in studies on the hormonal induction of diabetes. The large hormonal dosages used were those needed to induce glucosuria and were given for 5 days.

Adrenals: A commercial preparation of ZnACTH was found to be nearly 10 times more effective than a gel preparation of ACTH (ACTHAR) in augmenting the weight of adrenals, whereas both preparations increased equally the weights of kidney and liver. Bovine GH (BGH), which given alone had no effect on adrenal, kidney or liver weights, increased the effect of both ACTH preparations on adrenal weights. Although cortisone and dexamethasone given alone decreased adrenal weight, both increased the effectiveness of ACTHAR on adrenal weight. A further increase of adrenal weight was found in rats given a combination of ACTHAR + BGH with either cortisol or dexamethasone. These interactions deserve further study.

Thymus: ACTH, cortisol and dexamethasone reduced thymus weight 80% in 5 days.

Kidney: Cortisol (15 mg/day), dexamethasone (200 μg/day) and ACTHAR (100 IU/day) increased kidney weights by 50%. BGH injections decreased by one-half the amount of adrenal hormones needed to produce a given change in kidney weight. The stimulatory effect of adrenal hormones on kidney weight was also observed in rats bearing MtT-F4 transplantable pituitary tumors. There was a linear relation between adrenal weight and kidney weight in rats given ACTH, but the slope of the curve was dependent on the ACTH preparation given.

Liver: Liver weights were increased by ACTH, cortisol and dexamethasone. Although BGH was ineffective when given alone, it increased the effect of each of these hormones on liver weight.

In a preliminary experiment, rats of twenty different strains obtained from NIH Animal Production Unit were tested for susceptibility to the diabetogenic action of dexamethasone given at 3 dose levels for 5 days. Large differences in responsiveness to dexamethasone (blood glucose level and glucosuria) were found between strains. Organ weights also showed surprisingly large differences; certain strains failed to show organ weight responses while others did. When there were responses, the slopes varied. The large variation in organ weights and the differences in response are evidence of genetic influence on physiological activities regulated by hormones. This should be a fertile and important area for genetic studies. The organ weight differences should eventually be backed up by DNA, RNA and enzyme studies as well as hormone receptor, cyclic AMP and other studies.

Significance to Biomedical Research and the Program of the Institute: One of the major roles of the six hormones of the anterior pituitary gland is to modify or regulate the size and functional activity of other glands or tissues, which in turn control the overall metabolic state of the individual. This hormonal balance or imbalance controls how one feels and what one can do. Certain metabolic abnormalities and tumors are corrected by treatment with hormones or by removal of endocrine glands. Purified hormone preparations are needed for this work together with sensitive and quantitative tests which will estimate the concentration of hormones in the blood or transport

system and the rate of release of the hormones. One needs to know what factors control the biosynthesis and subsequent release of hormones by the pituitary gland.

The studies on induction of diabetes reveal the major role of hormonal interaction in a synergistic manner of at least three hormones. The extent of this synergism is such that hormonal induction of diabetes in man should be seriously investigated. The use of tolbutamide as an antidiabetic agent is contraindicated by our studies so far.

Proposed Course: Studies on the diabetogenic effects of hormones have been terminated due to the retirement of Dr. Bates in December 1973. Experiments have now been initiated to determine the mode of action of prolactin in the regulation of lipoprotein lipase in mammary gland and adipose tissue. Earlier studies in this laboratory showed that lipoprotein lipase activity is increased in mammary gland and decreased in adipose tissue during lactation and that these changes are induced and maintained through the secretion of prolactin by the pituitary gland.

Honors and Awards: None

Publications:

Bates, R.W.: Bioassay Methods for Prolactin in Blood. In Josimovich, J.B. (ed.): Problems in Reproduction: Fetal Nutrition and Lactation. New York, John Wiley and Sons. Vol. II. In press.

Bates, R.W. and Garrison, M.M.: Synergism Among Growth Hormone, ACTH, Cortisol and Dexamethasone in the Hormonal Induction of Diabetes in Rats and the Diabetogenic Effect of Tolbutamide. Endocrinology 93: 1109-1118, 1973.

Bates, R.W. and Garrison, M.M.: Hormonal Interactions Among GH, ACTH, Cortisol and Dexamethasone Upon Size of Kidney, Liver, Thymus and Adrenal. Proc. Soc. Exp. Biol. & Med. In press.

Bates, R.W. and Garrison, M.M.: Daily Changes in Concentration of Pancreatic and Serum Insulin and of Blood Glucose During 5 Days Treatment of Rats with Growth Hormone, ACTH, Cortisol, Dexamethasone and Tolbutamide Alone and in Combinations. Metabolism. In press.

Ben-David, M., Robard, D., Bates, R.W., Bridson, W.E., and Chambach, A.: Human Prolactin in Plasma, Amniotic Fluid and Pituitary: Identity and Characterization by Criteria of Electrophoresis and Isolectric Focusing in Polyacrylamide Gel. J. Clin. Endo. and Metab. 36: 951-964, 1973.

Goldfine, I.D., Kahn, C.R., Neville, D.M., Roth, J., Garrison, M.M. and Bates, R.W.: Decreased Binding of Insulin to its Receptors in Rats with Hormone Induced Insulin Resistance. Biochem. Biophys. Res. Comm. 53: 852-857, 1973.

Serial No. NIAMDD-LNE-5
1: Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Diabetes and Fat Metabolism

Previous Serial Number: SAME

Principal Investigators: Drs. Robert O. Scow and Sidney S. Chernick

Other Investigators: Dr. Margit Hamosh, Visiting Scientist
Dr. Hayden Klaeveman (NIAMDD-D & HD)
Dr. R. O. Wolff (NIDR-LOM)

Cooperating Units: NIAMDD-LNE-6
NIAMDD-D & HD
NIDR-LOM

PL 480 Agreement No. 02-009-1 with the
Department of Physiology, University of Skopje
Skopje, Yugoslavia

Man Years:

Total:	6-1/2
Professional:	2-1/2
Other:	4

Project Description:

Objectives: To determine influence of hormones and other factors on metabolism of fat and carbohydrate in liver, adipose tissue, mammary gland and other organs in normal and diabetic animals.

Methods Employed: Animals are deprived of one or several endocrine glands and treated with hormones or other substances. Effect of the above on metabolism of fat and carbohydrate in different organs and on blood hormone levels are determined with in vivo and in vitro studies using conventional and radioisotope techniques. Effects of hormones added in vitro are also studied.

Major Findings: Effect of hormones on lipid metabolism: Two independent processes are involved in mobilization of fatty acids from adipose tissue, lipolysis and fatty acid re-esterification. The former is a hormone activated process, usually involving cyclic AMP, and the latter is dependent on formation of alpha-glycerol phosphate from glucose. The effect of ACTH and adrenalectomy on these processes was studied in rat adipose tissue perfused in vivo. In-

jection of ACTH in normal animals increased lipolysis but not re-esterification, and resulted in increased mobilization of fatty acids from adipose tissue, whereas adrenalectomy decreased lipolysis and thereby reduced release of fatty acids. ACTH injection in adrenalectomized rats, however, increased both lipolysis and fatty acid re-esterification, and produced very little change in fatty acid release from the tissue. It is concluded that adipose tissue in adrenalectomized rats is sensitive to the lipolytic action of ACTH, but increased glucose utilization in the absence of glucocorticoid secretion enhances re-esterification and blocks release of FFA from the tissue. This probably explains the apparent ineffectiveness of ACTH in mobilizing fatty acids in adrenalectomized animals.

Pharyngeal (lingual) lipase: Earlier studies in this laboratory showed that serous glands in the tongue and pharyngeal tissues of the rat secrete a very potent enzyme which hydrolyzes triglyceride to mostly diglyceride and FFA at pH 4.5-5.5 and that this enzyme accounts for most of the lipolytic activity found in the stomach. Recent studies showed that a fatty acid-acceptor such as albumin is not necessary for the action of the enzyme and that FFA formed are retained, with diglyceride, in lipid droplets. Addition of albumin, but not Ca^{++}, enhances activity of the enzyme and results in complete hydrolysis of triglyceride to glycerol and FFA.

Studies in human volunteers have shown a similar lipolytic activity present in aspirates collected from the upper and lower esophagus and from the stomach. The lipolytic activity was not found in secretions collected from the major salivary glands (parotid, submaxillary and submandibular), thus excluding those tissues as a source of the enzyme. The findings indicate that pharyngeal tissues in man secrete a lipase which acts in the stomach to hydrolyze dietary triglyceride to partial glyceride and FFA. It is proposed that this is the first step in the digestion of dietary fat in man.

Metabolism of chylomicrons by lactating rat mammary gland: Perfused mammary glands of lactating rats take up triglyceride-fatty acids and -glycerol, and cholesterol from circulating chylomicrons. Hypophysectomy one day before perfusion lowered markedly both lipoprotein lipase activity and uptake of triglyceride and cholesterol in the tissue. Radioautographic studies of perfused tissue showed that the fatty acids and glycerol taken up from chylomicrons are readily incorporated into milk lipid. Radioautographic studies are now in progress to determine the fate of cholesterol taken up from chylomicrons.

Significance to Biomedical Research in the Program of the Institute: It has been generally thought that ingested fat is emulsified in the stomach by the "churning, kneading and squirting movements" of the stomach wall and that enzymatic digestion, by pancreatic lipase, does not begin until fat reaches the duodenum. Although several groups have reported that FFA are present in gastric contents soon after feeding fat, it was usually concluded that regurgitation of pancreatic secretions accounted for the lipolytic activity present in the stomach. The above findings in human volunteers, however, clearly show that pharyngeal tissues in man secrete a lipase which can act in

the stomach, at pH 4.5-5.5, to hydrolyze triglyceride to partial glyceride and FFA. It is proposed that dietary triglyceride is hydrolyzed to partial glycerides and FFA in the stomach by pharyngeal lipase and that this is the first step in the digestion of fat in man. Since fatty acids are poorly dissociated at the pH of stomach contents, they probably remain in lipid droplets while in the stomach. When the droplets enter the duodenum, the fatty acids are converted to soaps and, thus, contribute to emulsification of fat in the duodenum. The role of this process in the preparation of dietary fat for digestion by pancreatic lipase is under study.

Uptake of blood triglycerides by most tissues involves hydrolysis of triglyceride to FFA by lipoprotein lipase. Our earlier findings, in perfused adipose and mammary tissue, indicated that hydrolysis occurs stepwise, with partial glycerides and FFA formed at the luminal surface and in the endothelium of the capillary, and glycerol and FFA formed in the basement membrane area. Since lipoprotein lipase hydrolyzes only primary ester bonds it is unable to hydrolyze the resultant 2-monoglyceride to glycerol unless the glyceride is first isomerized to 1(3)monoglycerides. Thus, it is possible that lipoprotein lipase in capillary endothelium hydrolyzes triglyceride only to FFA and 2-monoglyceride, and that another enzyme, perhaps from periendothelial cells (pericytes), hydrolyzes 2-monoglyceride to FFA and glycerol. If uptake of triglyceride does involve two different enzymes, it is possible that partial glyceride could accumulate in endothelial cells whenever the activity of lipoprotein lipase exceeds that of monoglyceride lipase. Whether such an event can occur in the endothelium of either capillaries or large blood vessels is being studied.

Proposed Course: Adipose and mammary tissue will be perfused with emulsions of triglyceride containing labeled fatty acids in specific positions in order to determine more precisely where and how the triglyceride molecule is hydrolyzed after it is removed from blood. Emulsions of partial glycerides will also be perfused to determine if they can be utilized by the tissue and, if so, electron microscopic cytochemical studies will be made to localize where in the capillary wall the glycerides are hydrolyzed. Tissues will also be perfused with chylomicrons containing radioactive lecithin in order to study the fate of the surface film of chylomicrons.

Antibodies to purified lipoprotein lipase and to apoproteins of various plasma lipoproteins, especially VLDL, will be developed for measuring and localizing the enzyme and lipoproteins in blood and cells, and to study the interaction between circulating lipoproteins, capillaries, and lipoprotein lipase.

Studies will be continued to determine the role of pharyngeal lipase in fat digestion and to determine what factors regulate its synthesis and secretion. Studies will also be made to determine the source of the lipolytic activity found in esophageal aspirates from man. A collaborative project with the University of Oulu (Finland) will begin in December to determine the role of pharyngeal lipase in premature and neonatal infants. Isolation and purification of lipase from rat tongue will also be initiated.

Honors and Awards: None

Scow, R.O. (Editor): Endocrinology, Proceedings of the Fourth International Congress of Endocrinology. Amsterdam, Excerpta Medica. 1973. International Congress Series No. 273. 1280 pp.

Scow, R.O., Blanchette-Mackie, E.J., Mendelson, C.R., Hamosh, M. and Zinder, O.: Incorporation of dietary fatty acids into milk triglyceride: Mechanism and regulation. In: Modern Problems in Paedictrics. Basal, S. Karger. Vol. 15, No. 3. In press.

Scow, R.O., Mendelson, C.R., Zinder, O., Hamosh, M. and Blanchette-Mackie, E.J.: Role of lipoprotein lipase in the delivery of dietary fatty acids to lactating mammary tissue. In: Galli, C., Jacini, G. and Pecile, A. (Eds.) Dietary Lipids and Postnatal Development. New York, Ravin Press. 1973, pp. 91-114.

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Serial No. NIAMDD-LNE-6
1. Nutrition and Endocrinology
2. Endocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Transport of Lipid, Hormones and Enzymes through Cells and Membranes

Previous Serial Number: SAME

Principal Investigators: Drs. E. Joan Blanchette-Mackie and Robert O. Scow

Other Investigators: Gloria Devore, Guest Worker

Cooperating Unit: NIAMDD-LNE-5

PL 480 Agreement #06-101-1 with the Department of Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem, Israel

Man Years:

Total:	2-1/2
Professional:	1-1/2
Other:	1

Project Description:

Objectives: To study structure and metabolism of cells that synthesize and secrete plasma lipid and hormones. To study transport of lipid, hormones and enzymes across the capillary endothelium, basement membrane and extracellular space in different kinds of tissues. To study the uptake of lipid, hormones and enzymes by cells. To correlate structural and biochemical changes induced in cells and tissues by various means.

Methods Employed: Animals of different ages and in various physiological states (fasting, pregnant, lactating, diabetic) are used to study with biochemical and morphological techniques the synthesis and secretion of lipoprotein triglyceride by liver and intestines, and the utilization of plasma triglyceride by adipose tissue, mammary gland and muscle. Perfused adipose and mammary tissues are used to localize with cytochemical techniques the sites of action of lipoprotein lipase and the mode of transport of fatty acids between blood and tissue cells. Perfused tissues are also used to determine with immuno-cytochemical techniques the distribution and site of synthesis of lipoprotein lipase and the transport of hormones across capillary endothelium and extracellular space. Isolated chylomicrons and purified lipoprotein lipase incubated *in vitro* are used to study morphological changes in chylomicrons induced when the triglyceride core is reduced by lipolysis.

Major Findings: Effects of purified lipoprotein lipase on chylomicrons: Chylomicrons are particles in blood which carry fatty acids in the form of triglyceride from intestine to other tissues in the body. They consist of a core of triglyceride, with traces of cholesterol ester, enclosed by a surface film of phospholipid (mostly lecithin), cholesterol and protein. Our earlier studies in perfused adipose tissue and mammary gland showed that chylomicron triglyceride was hydrolyzed by lipoprotein lipase in the capillary endothelium to free fatty acids (FFA) and partial glycerides. The FFA produced were released to the blood stream, while the partial glycerides were taken into vacuoles and microvesicles of the endothelium for further hydrolysis and transport across the capillary wall. The rest of the chylomicron, the remnant, consisting of surface film and residual core lipid, was released to the blood stream.

The interaction of lipoprotein lipase with chylomicrons was studied in vitro using highly purified enzyme isolated from bovine milk. It was found that lipoprotein lipase readily hydrolyzed chylomicron triglyceride to FFA and glycerol when sufficient albumin was present in the medium to bind the FFA produced. However, if excess albumin was present, 4-8 times that needed to bind FFA (6 mol/mol albumin), two-thirds of the monoglyceride formed was removed and trapped outside of the chylomicrons, and remained unhydrolyzed. Recent studies in Sweden have shown that lipoprotein lipase hydrolyzes only the 1(3) ester bonds of glycerides and that complete hydrolysis of triglyceride by the enzyme, to FFA and glycerol, requires (nonenzymatic) isomerization of 2-monoglyceride to 1(3)-monoglyceride. Thus, it is possible, in our experiments, that the presence of excess albumin in the medium blocked isomerization of some of the monoglyceride formed.

Electron microscopic studies reported earlier showed that chylomicrons are enveloped by a monolayer surface film. The surface film was visualized after the chylomicron core had been reduced by action of lipoprotein lipase from postheparin plasma. These studies have been extended using purified lipoprotein lipase to determine early changes induced in chylomicron structure by the enzyme. It was found that FFA and water accumulated within chylomicrons if the incubation medium did not contain sufficient albumin to accept the FFA produced. Retention of FFA and water in chylomicrons was verified with scanning electron microscopy. The morphological appearance of chylomicrons containing FFA was markedly affected by pH during preparation for microscopy. Circular aqueous spaces were present in the core of specimens prepared at pH 7.4-8.1 whereas water was absent and fatty acids formed lamellae in the core of specimens prepared at pH 5.5. Studies using the freeze etching technique indicated that the surface film of chylomicrons was also changed by the action of lipoprotein lipase.

Transport of chylomicron-fatty acids across mammary capillary endothelium: Electron microscopic cytochemical studies showed that lipoprotein lipase activity is present in the endothelial cells of capillaries in lactating mammary gland, and that glyceride derived from chylomicrons is transported across the capillary endothelium in microvesicles and vacuoles.

Significance to Biomedical Research and the Program of the Institute: Alterations in the metabolism of plasma triglyceride occur in various hyperlipemic states. In some, synthesis and secretion of lipoproteins are abnormal, whereas in others, uptake is reduced, sometimes with changes in lipoprotein lipase activity. Localization of lipoprotein lipase synthesis and activity in tissues and study of the interaction of lipoprotein lipase with chylomicrons and other lipoproteins of plasma will provide valuable information concerning factors that regulate removal of triglyceride from the blood stream.

Proposed Course: We plan to use the freeze etching technique, combined with electron microscopy, to study in detail the interaction of lipoprotein lipase with chylomicrons, the transport of lipid from blood to tissue cells, and the secretion of lipid by cells. The freeze etching technique is especially suitable for these studies because it provides surface views of the interphase between layers of lipid molecules, as in cell membranes and between the surface film and core of chylomicrons. In addition, the technique preserves lipid because it does not require chemical dehydration of specimens. We wish to localize lipoprotein lipase on the surface of chylomicrons and to study the effect of the enzyme on the surface film and the accumulation of fatty acids in the core. We also wish to localize lipoprotein lipase in the capillary, study the interaction of chylomicrons with capillary plasma membrane, determine sites of lipolysis and transfer of glyceride from chylomicrons to endothelial cells, and study transport of lipid across capillary endothelium.

Immuno-cytochemical techniques will be developed for localizing lipoprotein lipase. They will be used to study distribution of the enzyme in mammary and adipose tissue during various physiological states (e.g. pregnancy, lactation, fasting), to localize the enzyme in capillary endothelium, and to study the interaction of the enzyme with chylomicrons.

The composition and structure of chylomicron remnants, produced in vivo and in vitro, and the effects of phospholipases on surface film of chylomicrons will be studied using chylomicrons containing ^{3}H-cholesterol, ^{32}P-lecithin and ^{14}C-oleic acid.

Honors and Awards: None

Publications:

Blanchette-Mackie, E.J. and Scow, R.O.: Effects of lipoprotein lipase on the structure of chylomicrons. J. Cell Biol. 58: 689-708, 1973.

Scow, R.O., Blanchette-Mackie, E.J., Hamosh, M. and Evans, A.J.: Catabolism of chylomicrons. Wiss. Veröffentl. Deut. Ges. Ernährung. 23: 100-114, 1973.

Serial No. NIAMDD-LNE-7
1. Nutrition and Endocrinology
2. Membrane Regulation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanism of Action of Hormones on Adenylate Cyclase Systems

Previous Serial Number: SAME

Principal Investigator: Dr. Martin Rodbell

Other Investigators: Dr. Michael Lin, Staff Fellow
Dr. Yoram Salomon, Visiting Fellow
Dr. Constantine Londos, Visiting Associate
Dr. Michael Schramm, Visiting Scientist
Dr. Bruce R. Martin, International Post-doc. Fellow

Cooperating Units: Dr. Victor Hruby, University of Arizona

Man Years:

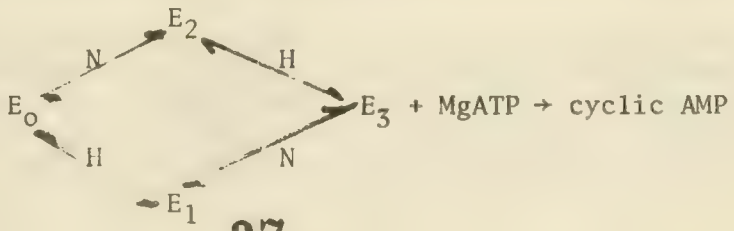
Total:	4
Professional:	3
Other:	1

Project Description:

Objectives: To determine the mechanism of action of various hormones on adenylate cyclase systems in membranes from various animal cells.

Methods Employed: Cell membranes from a variety of cell types are purified by differential centrifugation in sucrose gradients. Adenylate cyclase activity, binding of labeled hormones and nucleotides were carried out by procedures developed in this laboratory. Various types of chromatographic procedures, solid-phase synthesis of peptide hormones, and methods for measuring metabolism of nucleotides were also employed.

Major Findings: I. Regulation of adenylate cyclase systems. A. Mechanism of guanine nucleotide action in animal cells: Previous studies have shown that GTP and glucagon activate the glucagon-sensitive adenylate cyclase system by a concerted or interdependent process. Kinetic data of binding and action of the hormone under varying conditions of GTP concentration have been computer fitted to the following model:



in which the enzyme system (E) exists in four states, in equilibrium, which are formed in response to the binding of glucagon (H) and GTP(N) to the enzyme system; state E_3 is the high activity form of the enzyme system.

Gpp(NH)p (5' guanylylimidodiphosphate) has been found to substitute for GTP in the process of activation of adenylate cyclase. The synthetic analogue is not hydrolyzed at its terminal phosphate by nucleotidases in membranes. In contrast to GTP, Gpp(NH)p causes marked activation, in the absence of hormones, of all adenylate cyclase systems in all eucaryotic cells investigated in this laboratory.

Some of the characteristics of Gpp(NH)p activation observed with all systems investigated, including adenylate cyclase from liver, fat cells, adrenal and thyroid cells, and from avian, fish, and amphibian erythrocytes, are as follows: Gpp(NH)p activates these systems by a time-dependent process exhibiting transient lags of one to 3 min; lag times are proportional to the reciprocal of nucleotide concentration and are reduced by increasing reaction temperatures. In most systems the optimal temperature is between 35 and 40°. Addition of the hormones operating on the various systems abolishes the lag in onset of Gpp(NH)p activation. This finding is the mirror image of previous kinetic studies which showed that glucagon in the presence of limiting concentrations of GTP activates by a time-dependent process which is abolished by addition of maximal concentrations of GTP. The characteristics of Gpp(NH)p activation add further evidence for the reaction scheme illustrated above and indicate that the activation of the enzyme requires binding of guanine nucleotides to a regulatory site. Hormones appear to regulate this process by increasing the rate of formation of state E_3.

The finding that Gpp(NH)p activates all adenylate cyclase systems irrespective of the nature of the receptors linked to these systems suggests that the nucleotide regulatory site is separate from the receptors and may be associated with the catalytic component. This is further suggested by the finding that adenylate cyclase from mouse adrenal tumor lacking a functional ACTH receptor responds to Gpp(NH)p. Moreover, solubilization of the hepatic glucagon-sensitive adenylate cyclase with a neutral detergent (Lubrol) followed by removal of detergent results in a completely soluble form of the enzyme that is activated by Gpp(NH)p but not by the hormone. The soluble enzyme has been partially purified (about 50-fold).

Gpp(NH)p labeled with ^{3}H or ^{32}P binds to hepatic membranes with a dissociation constant of about 10^{-7} M. GTP binds to these sites with five-fold higher affinity. GDP inhibits binding of GTP and Gpp(NH)p with a K_i of about 10^{-6} M. GTP and GDP also inhibit activation of the hepatic adenylate cyclase system by Gpp(NH)p with the same relative affinities.

GDP inhibits both basal and Gpp(NH)p activities whereas GTP activates slightly in the absence and strongly in the presence of hormones. These findings suggest that the terminal phosphate is necessary for activation. Gpppp and ppGpp do not bind to the nucleotide regulatory sites based on findings that these substances do not activate adenylate cyclase or inhibit

activation by Gpp(NH)p; 5' GMP and cyclic GMP also do not affect adenylate cyclase activity.

The different potencies of GTP and Gpp(NH)p in spite of nearly equivalent affinities for the nucleotide regulatory site raises the possibility that the terminal phosphate of GTP is utilized or hydrolyzed at this site. This possibility will be investigated when the soluble form of the enzyme has been purified from non-specific nucleotide phosphohydrolases.

In view of the obligatory role of the guanine nucleotide regulatory site in the activity and response of adenylate cyclase systems to hormones, the effects of combinations of saturating concentrations of hormones on the multi-receptor fat cell adenylate cyclase system has been re-investigated. Previous studies have shown that combinations of saturating concentrations of glucagon, secretin, ACTH, and epinephrine does not lead to additive responses; activities were usually equivalent to that given by the most active hormone in the combination. This was taken as evidence that the fat cell system consists of a common catalytic unit which interacts with multiple receptors. We have found that, under conditions of limiting concentrations of GTP, the combined hormones give additive responses. Addition of Gpp(NH)p or GTP abolishes additivity since the hormones and nucleotides activate the enzyme in a cooperative fashion. Additivity suggests that the fat cell system is a multi-subunit enzyme; the receptors for the individual hormones may be linked in a structurally specific manner with each of the subunits. Studies of the kinetics of substrate utilization by the fat cell system suggest also that the enzyme is a multi-site enzyme since kinetic analysis shows a high degree of negative cooperativity suggestive of a linked subunit process. Gpp(NH)p at maximal stimulating concentrations abolishes the cooperativity of substrate utilization and converts the enzyme to a state having nearly equivalent activity to that observed with the combined hormones and sub-maximal concentrations of the nucleotide.

B. Kinetics of substrate utilization: A number of studies have shown that hormones increase the Vmax of substrate utilization by adenylate cyclase systems without altering the affinity constant. A divalent cation, preferentially Mg^{2+}, is required for activity, which suggests that the reactive substrate is $MgATP^{2-}$. ATP in excess of magnesium ion inhibits adenylate cyclase activity. Several studies have also shown that an excess of magnesium ion over ATP results in marked enhancement of basal (no hormone added) activity. In the presence of hormones, lower concentrations of magnesium are required for enhanced activity. One interpretation of this finding is that hormones increase the affinity of the active site for Mg^{2+}. Another possibility is that a species of substrate (ATP^{4-} or $HATP^{3-}$) uncomplexed with magnesium ion has a high affinity for the active site and competitively inhibits MgATP binding at this site.

Both ATP and App(NH)p are substrates for adenylate cyclase. The terminal phosphate of ATP has a lower affinity for protons ($pK_A = 7.1$) than that of App(NH)p ($pK_A = 7.7$). Studies of the pH dependency of adenylate cyclase systems with the two substrates showed that the pH optimum is

shifted to higher pH with App(NH)p as substrate which suggests that the protonated species ($HATP^{3-}$ or $HApp(NH)p^{3-}$) is an inhibitor. Studies of the effects of increasing magnesium ion concentration at different pH showed that activity correlates with the concentration of protonated species of substrate present in the incubation medium. Activation of the enzyme system by hormone plus GTP or by Gpp(NH)p alone results in states which have lower affinities for the protonated species. It appears, therefore, that the activating ligands alter the conformation and possibly charge distribution at the active site with resultant lowering of the affinity of the protonated species and increases in the reactivity of MgATP at this site.

C. Role of sulfhydryl groups: Adenylate cyclase activity is inhibited by a number of sulfhydryl reagents. ATP or MgATP protect against sulfhydryl inhibition which suggests that a critical sulfhydryl group is located at the active site of the enzyme. GTP or Gpp(NH)p do not protect against inhibition by sulfhydryl agents. It has been reported that glucagon increases the susceptibility of sulfhydryl inactivation which raises the possibility that hormones (or guanine nucleotides) increase the reactivity of a crucial sulfhydryl group involved in catalysis. In contrast, fluoride ion, a potent stimulator of all adenylate cyclase systems in animal cells, protects against sulfhydryl inhibition. This effect of fluoride is suggestive evidence that the halide reacts with the active site of the enzyme.

II. Structure-function relationships in glucagon and secretin: In collaboration with Dr. Victor Hruby of the Department of Chemistry at the University of Arizona, we have investigated the biological activity and receptor binding of glucagon when it is chemically modified by a variety of procedures. Des-His-1-glucagon, in highly purified form, retains from 5 to 10 percent of the binding and actions of native glucagon. Removal of two amino acids from the carboxy-terminal end of the molecule and replacement of methionine with homoserine results in nearly complete loss of binding and action of the hormone.

Solid-phase synthesis of secretin has proven not to be feasible primarily because of the large percentage of non-biologically active species formed by this procedure and the inability to purify the small amount of active material. A new procedure has been developed which combines solid-phase synthesis of certain fragments and coupling by classical synthetic procedures of these fragments. This procedure seems promising. The ultimate goal of this project is to synthesize various analogues containing amino acid replacements thought to be crucial for biological activity.

Significance to Biomedical Research in the Program of the Institute: Adenylate cyclase is a key regulatory enzyme system for the initial actions of a number of peptide hormones, biogenic amines, and prostaglandins. The discovery that the enzyme system contains a nucleotide regulatory site, specific for GTP, which regulates the activity and response of the enzyme to hormones has given new insights into the molecular basis for the regulation of this important enzyme system. The possibility that the terminal phosphate of GTP is utilized at this site raises the question of whether other sub-

stances may regulate the utilization of GTP at the regulatory site. Agents such as adenosine, long known to alter adenylate cyclase activity and metabolism of cells, may affect this process. The discovery that a synthetic analogue of GTP, Gpp(NH)p, activates a number of adenylate cyclase systems in animal cells irrespective of the nature of the receptor and independently of hormones provides the means for exploring the physico-chemical nature of adenylate cyclase systems and the process by which guanine nucleotides activate the enzyme.

Proposed Course: Efforts will be made to isolate and characterize the various components of adenylate cyclase systems in cells from liver, adrenal, fat, and from frog erythrocytes. Partial success has been achieved, using Gpp(NH)p as activator, in purification of a soluble form of the liver adenylate cyclase system. When sufficiently purified, binding and metabolism of guanine nucleotides by the enzyme will be investigated in addition to studies of its chemical properties (subunits, molecular size, etc.). Increased emphasis will be placed on isolation of the glucagon receptor using the purified enzyme as a tool for assaying a functional receptor. Further studies of the structure-function relationships in glucagon and secretin are planned.

Honors and Awards: Awarded Jacobeaus Prize by Scandanavian Endocrine Society.

Publications:

Rodbell, M., Lin, M.C., and Salomon, Y.: Evidence for interdependent action of glucagon and nucleotides on the hepatic adenylate cyclase system. J. Biol. Chem. 249: 59-65, 1974.

Salomon, Y., Londos, C., and Rodbell, M.: A highly sensitive adenylate cyclase assay. Anal. Biochem. 58: 541-548, 1974.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical Studies Related to Nutrition

Previous Serial Number: NIAMDD-LNE-8

Principal Investigators: Drs. J. G. Bieri, J. N. Williams, Jr., and H. C. Windmueller

Other Investigators: Dr. R. E. Poukka Evarts, S. L. Thorp and A. E. Spaeth

Cooperating Units: Dr. W. T. London, DVM, NINDS
Dr. P. M. Farrell, MD, NIAMDD
Dr. P. Herbert, NHLI

Man Years
Total: 7
Professional: 5
Other: 2

Project Description:

Objectives - To determine the nutritional, biochemical and physiological role of essential nutrients for experimental animals. To define the metabolic function of certain nutrients and to study nutritional effects on the tissue levels of various metabolites.

Methods Employed - Rats, hamsters and guinea pigs are fed specially prepared, highly purified diets that contain adequate amounts of each nutrient known to be required by the particular species. The effects of specific deficiencies and imbalances are assessed by measurement of physiological, chemical and enzymological changes in the animal, its tissues and excreta.

The relationship of nutrition to other scientific areas, such as neurology and pathology, is studied.

Major Findings

A. Metabolism and Function of Fat-Soluble Vitamins (J. G. Bieri & R. Poukka Evarts)

Some nutritionists have postulated that the increased consumption of vegetable oils in the U.S. in recent years could lead to an unsatisfactory

vitamin E status in significant segments of the population. Studies to test the vitamin E adequacy of the primary edible oils, soybean and corn oils, and also of safflower oil, have been conducted in rats. The oils were fed as 20% by weight of the diet for as long as 26 weeks. Assessment of vitamin E status by in vitro red cell hemolysis, plasma creatine phosphokinase activity or testicular histology indicated that all three oils provided adequate vitamin E. Analyses of tissues for their content of polyunsaturated fatty acids (PUFA) and tocopherols showed that the molar ratio of PUFA:α-tocopherol ranged from 1000-3000; heart and lung had the most favorable (lowest) ratio, muscle and testis were intermediate and liver and kidney were highest. A satisfactory ratio for red cell membranes was 2000.

The collaborative study with Dr. W. T. London (NINDS) on the protein and calorie requirements of pregnant rhesus monkeys has shown that 2.1 grams of protein and 88 kilocalories per kg. of body weight permitted normal pregnancies with normal babies. When 1.2 grams of protein and 50 kilocalories were fed, birth weights were highly variable with some infants much smaller while others were heavier than normal.

Analyses for α-tocopherol in autopsy tissues from children with cystic fibrosis (Dr. P. M. Farrell, Pediatric Metabolism Branch) showed that the vitamin was extremely low. Red cell and plasma analyses from CF children under treatment and dosed with water dispersible vitamin E showed almost normal levels of the vitamin, but a decreased red cell linoleic acid content indicated continued impairment in fat utilization.

B. Glutamine Metabolism by Intestine; Lipoprotein Characterization

Recently we found that the small intestine continuously extracts large amounts of glutamine from the blood and metabolizes it to CO_2, several organic acids and other amino acids. In the rat, arteriovenous measurements across other large organs or body regions, including liver, kidneys, lungs, heart, head or hindquarter, revealed no other tissue that extracted such large quantities of glutamine. Thus, intestine is a major site for the metabolism of glutamine released into the circulation by other tissues. Glutamine uptake by intestine is sufficient to cause a turnover of the entire plasma glutamine pool every 4.6 min. Several approaches were used to get more insight into this process. By modifications in the isolated, vascularly perfused rat intestine preparation developed in this laboratory, it became possible to determine directly the rate of glutamine uptake from blood and the rate of metabolism of glutamine carbon to individual products. Significant findings were that (a) 32% of the CO_2 produced by the intestine was derived from metabolism of plasma glutamine, indicating the importance of this amino acid as a respiratory substrate for this organ, and (b) within 10 min after blood containing [^{14}C]glutamine of constant specific activity was perfused without recirculation through intestine, the rate at which ^{14}C-labeled products appeared in intestinal venous blood reached a steady-state, reflecting the high turnover rate for glutamine in this tissue. Only a small fraction of

the glutamine carbon taken up from arterial blood by intestine was incorporated into protein (14%) and the rest reappeared in venous blood as CO_2 (57%), citrulline (6%), proline (5%) and organic acids (18%), predominantly citrate and lactate. Glutamine nitrogen taken up could be approximately accounted for by the quantities of citrulline (34%), alanine (33%), ammonia (23%) and proline (10%), released into intestinal venous blood. Studies with [^{14}C]glucose indicated that plasma glucose is an important carbon source for the alanine produced. Tissue analyses following perfusion with [^{14}C]glutamine in vivo revealed the highest uptake in the small intestinal mucosa. Attempts are underway to identify more precisely the cells responsible for the uptake by autoradiographic analysis of frozen tissue slices following [^{14}C]glutamine perfusion. A hypothesis explored and rejected was that ammonia derived from glutamine metabolism might serve to neutralize acids in the intestinal lumen. The rate of ammonia influx into the intestinal lumen was found to be no greater when the lumen was perfused with a salt solution at pH 2 than at pH 7, although the pH 2 solution was 80% neutralized in its transit through the lumen. Lumen and intestinal venous blood ammonia concentrations were found to be similar. Elevating the blood ammonia levels by intravenous infusion led to an immediate increase in the luminal ammonia concentration, indicating a rapid equilibrium between ammonia in the blood and the intestinal lumen.

In another project, in collaboration with Dr. P. Herbert (NHLI), work is continuing on the isolation and characterization of the plasma and intestinal lymph lipoprotein apoproteins of the rat, such information being essential for the interpretation of physiological lipoprotein studies in this species. Five peptides which comprise the group of small apoproteins of plasma high density lipoproteins were isolated. Their amino acid composition, amino and carboxyl end-groups, carbohydrate content, and capacity to activate lipoprotein lipase suggest a striking analogy to corresponding apoproteins in human lipoproteins. Work is also underway to identify the apoproteins that accumulate in hepatic liposomes of orotic acid-fed rats where the production of very low density lipoprotein is totally blocked.

C. Biochemical Studies of Cytochromes and Sub-cellular Particles

Studies have centered around the biochemical characterization of mitochondrial populations and submitochondrial fractions. Density gradient centrifugation of highly purified rat liver mitochondria yielded a fraction containing lysosomes and peroxisomes and which exhibited a bimodal distribution on a linear sucrose density gradient. Enzyme evidence indicated that these less dense lysosomes and peroxisomes were not fragments of the more dense particles but were true sub-populations. In other experiments, six sub-fractions of purified mitochondria were obtained by centrifugation and and representative enzymes and constituents assayed. Specific activities of cytochrome oxidase, monoamine oxidase and malate dehydrogenase did not vary among the fractions. There were, however, significant gradient changes in activities of adenylate kinase and glucose-6-phosphatase, and in cytochrome c concentration. It was concluded that some of these subparticles may represent

mitochondria whose outer membrane is still contiguous with the endoplasmic reticulum.

Studies on the binding of cytochrome c to the outer and inner mitochondrial membranes indicate that the outer membrane possesses many more binding sites than the inner membrane. This is unusual since it is thought that cytochrome c functions only in the inner membrane. Injection of glucagon into rats for 2 days caused a sharp drop in mitochondrial population in the liver.

Proposed Course of the Project

Clarification of nutritional responses and explanations of interrelationships at the biochemical level remain the primary goals of the project.

Publications

Bieri, J. G.: Fat-soluble vitamins in the eighth revision of the Recommended Dietary Allowances. J. Am. Dietetic Asso. 64, 1974.

Bieri, J. G. & Evarts, R. Poukka: Vitamin E activity of γ-tocopherol in the rat, chick and hamster. J. of Nutrition: in press.

Bieri, J. G.: Recommended Dietary Allowances--Vitamin A; vitamin E: Recommended Dietary Allowances, Eighth Edition. Washington, D.C., National Academy of Sciences, in press.

Bieri, J. G. and Evarts, R. Poukka: Gamma Tocopherol: Metabolism, biological activity and significance in human vitamin E nutrition. J. Am. Clin.Nutrition: in press.

Windmueller, H. G. and Spaeth, A. E.: Uptake and metabolism of plasma glutamine by the small intestine. J. Biol. Chem.: in press.

Herbert, P. N., Windmueller, H. G. Bersot, T. P., and Schulman, R.: Characterization of the rat apoproteins: I. The low molecular weight proteins of rat plasma high density lipoproteins. J. Biol. Chem.: in press.

Krauss, R. M., Windmueller, H. G., Levy, R. I. and Fredrickson, D. S.: Selective measurement of two different triglyceride lipase activities in rat post-heparin plasma. J. Lipid Res. 14: 286-295, 1973.

Eisenberg, S., Windmueller, H. G., and Levy, R. I.: The metabolic fate of rat and human lipoprotein apoproteins in the rat. J. Lipid Res. 14: 446-458, 1973.

Williams, J. N., Jr. and Thorp, S. L.: Studies on the polymodal distribution of hepatic lysosomes and peroxisomes. Proc. Soc. Exp. Biol. & Med. 145, 289-293, 1974.

Serial No. NIAMDD-LNE-9
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure and function of an extracellular ribonuclease (barnase) and its intracellular inhibitor (barstar) from *Bacillus amyloliquefaciens*.

Previous Serial Number: SAME

Principal Investigator: Dr. Robert W. Hartley

Other Investigators: None

Cooperating Units: Large-scale laboratory, Office of Chief, LNE, NIAMDD. Collaboration with D. M. Blow, and R. C. Sheppard, (Laboratory of Molecular Biology, Medical Research Council, Cambridge, England.

Man Years

Total:	2.0
Professional:	1.0
Other:	1.0

Project Description:

Objectives: To further characterize and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its product enzyme-inhibitor pair may be applied to a broad study of structure, function and synthesis.

Methods Employed: *B. amyloliquefaciens* (strain H and derivatives and several other strains all of which were formerly considered to be strains of *B. subtilis)* are grown in synthetic media in a variety of batch and continuous flow fermentations. Isolation of barnase involves: 1) adsorption onto P-cellulose from acidified culture medium, followed by stepwise elution, 2) desalting by Sephadex chromatography, 3) gradient chromatography on CM-cellulose. The inhibitor (barstar) is extracted from the previously acidified and acetone extracted cells at pH 8.5. It is purified by acid precipitation, DEAE-cellulose and Sephadex G-75 chromatography and by making use of its specific binding to purified enzyme.

Work on the determination of amino acid sequence involves amino acid analysis, enzymatic digestions, peptide separations, and peptide sequencing by the combined Edman-dansyl techniques. Amino acid analysis is by automated liquid

chromatography using a modified JEOLCO analyzer equipped for detection with fluorescamine. Dansyl amino acid derivatives are identified by thin layer chromatography on polyamide layers. Peptide separations are obtained by Sephadex and ion-exchange chromatography.

The reversible transitions undergone by barnase and barstar and their derivatives under the influence of temperature changes or denaturing agents are studied primarily by ultraviolet and fluorescence spectroscopy, but also by optical rotatory dispersion, circular dichroism, and exclusion chromatography, with the use of proteolytic enzymes and by equilibrium binding studies, using radioactive denaturing reagents. These techniques are also used to study complementation reactions between natural and synthetic peptide sequences of the two proteins as well as conformational properties of each peptide alone. Studies of the peptide derivatives also include use of antibodies, chromatography, crystal growth, etc.

Equilibrium ultracentrifugation has been used to measure the molecular weights of the enzyme, the inhibitor, and their complex, and to determine the molecular weight homogeneity of each. Disc and equilibrium pH gradient electrophoresis have also been used to assess homogeneity and to demonstrate the reversibility of complex formation. Bacterial mutants are obtained by chemical mutagenesis of vegetative cells and by gamma irradiation of spores. A variety of techniques are used to isolate ribonuclease, protease, amylase, nutritional and antibiotic resistant mutants.

By their addition to appropriately prepared cells, radioactive amino acids have been incorporated into barnase and into nascent barnase peptides.

Crystals of barnase are grown in hanging drops (10 µl) suspended over suitable solvents. Isomorphous crystals containing heavy atoms are prepared by soaking crystals in solutions of appropriate heavy atom compounds. The three dimensional structure of barnase is being determined by methods of X-ray crystallography.

The Merrifield procedure has been used to synthesize portions of the barnase sequence.

Major Findings: The transition induced in barnase by sodium dodecyl sulfate (SDS), has been studied, using fluorescence of the tryptophane chromophore as a conformational probe. Two theoretically independent procedures, one based on simple reagent conservation, the other on the theory of complex equilibrium. Both yield a difference in SDS binding of about 13 moles per mole between the native and the SDS-transformed barnase. This result is very strong evidence that the transition may be treated as a "two-state" reaction, in which virtually all molecules (at equilibrium) are either in the native or the completely transformed state. The first procedure also shows that the native molecule binds little if any SDS. This last conclusion has been confirmed by direct binding studies, using 35S-labeled SDS. Preliminary results for the transformed molecule using this technique suggest that it binds about 20 SDS molecules in the transition region. This conflicts with the results of the optical titration experiments above and further experiments are indicated.

A JEOLCO amino acid analyzer has been modified and miniaturized to take advantage of the new fluorescamine reagent. Its sensitivity is now such that samples as small as 50 pM per residue can be analyzed. A very promising modification is currently under investigation by which a separate buffer stream in the detection system is eliminated by including organic buffers in the fluorescamine solvent. So far, the sensitivity obtained by this scheme is much less than that of the conventional procedure, but this may be due to suboptimal choice of buffers and to difficulties in getting them pure.

The use of one very good isomorphous replacement and two rather poor ones have so far proved insufficient for the resolution of barnase structure at 2.5 Å resolution by X-ray diffraction.

A semi-quantitative assay for the barnase-barstar complex has been developed taking advantage of the greater susceptibility of barstar to disruption by cationic detergents. This involves assaying for barnase after exposure of the sample to pronase in the presence of an appropriate level of cetyltrimethylammonium bromide (CETAB). This assay will greatly facilitate the isolation of both the complex and barstar.

An improved synthetic bacterial growth medium has yielded 3-4 fold increases in production of both barstar and barnase in a small fermentor. Problems in scale-up to pilot-plant scale involve difficulty in supplying enough oxygen in the big fermentor.

Significance to Bio-Medical Research and the Program of the Institute: The small size, stability, easily measured enzyme activity and absence of disulfide bridges in barnase made it an excellent choice for a long-range study of the relation between the sequence of an enzyme and its structure and function. The inhibitor is an even simpler protein and should be useful in a similar fashion. Together, they offer a model system for the study of a protein-protein interaction.

The nature and extent of the thermal transition of barnase gives strong support to the concept that the three-dimensional structure of a protein may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermodynamic analysis of the factors involved in maintaining a folded protein structure.

The production of both of these proteins by the same organism is also of biological interest.

It has been suggested that protein-detergent interactions may serve as models for interactions between proteins and phospholipids in biological membranes.

Proposed Course of Project: Collaboration with the workers in Cambridge on the X-ray solution of barnase structure and on peptide complementation studies will continue.

Sequencing of barstar and attempts to grow crystals of barstar and the complex will continue.

Efforts to improve yields, particularly of barstar and complex, will continue.

The nature of the barnase-SDS interaction will be further investigated and extended to an investigation of the reaction between barnase and cationic detergents.

Honors and Awards: NONE

Publications:

Hartley, R. W. and Smeaton, J. R.: On the reaction between the extracellular ribonuclease of *Bacillus amyloliquefaciens* (Barnase) and its intracellular inhibitor (Barstar). J.Biol. Chem. 248: 5624-5626, 1973.

Serial No. NIAMDD-LNE-10
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on structure and function of nucleic acids.

Previous Serial Number: SAME

Principal Investigator: Dr. G. W. Rushizky

Other Investigators: Mr. J. E. Mozejko, Mr. D. L. Rogerson, Jr., and Dr. H. A. Sober, LNE, NIAMDD

Cooperating Units: Large-Scale Laboratory, LNE, NIAMDD

Man Years

Total:	2.3
Professional:	2.3
Other:	0

Project Description:

Objectives: To study the enzymology and chemistry of nucleic acids and to develop new methods for the determination of nucleotide sequences in RNA and DNA.

Methods Employed: Various procedures for the preparation and fractionation of large oligonucleotides derived from RNA (MS2 virus) and DNA (fd virus) were investigated.

1) The nuclease S1 was prepared from dried mycelia of *Asp. oryzae* as well as commercial amylase powder. The enzyme was used to hydrolyze *E. coli* phage RNA and DNA and the digestion products were examined by sucrose sedimentation, gel electrophoresis, and chromatography on paper or DEAE-cellulose.

2) As an aid for quantitation of hydrolysis products as described in #1, ^{32}P and ^{33}P-labeled viral nucleic acids were prepared.

3) Two enzymes from a related strain of *Asp. oryzae* were investigated which only cleave DNA, and yield predominantly oligomers of size greater than the hexamer.

Major Findings : S1 was prepared according to the procedure of Vogt (Eur. J. Biochem. 33, 192, 1972) and found to be contaminated with RNase T2 which has a similar molecular weight (about 30,000) and pH optimum (4.5) as S1, but no Zn requirement for enzymatic activity. Passage of contaminated S1 through CM-cellulose removed all extraneous DNase and RNase T2 activity.

This was confirmed by the stability (23°, 3 hrs) of MS2, fd nucleic acids and trinucleotides in the absence of Zn at enzyme:substrate ratios (E) of 1, conditions where addition of Zn at 1 mM would have produced complete hydrolysis of all substrates to nucleoside 5'-monophosphates. Purified S1 enzyme showed no specific base preference in the hydrolysis of MS2 RNA or fd DNA and was found to be useful for the determination of nearest neighbor base compositions. Partial S1 hydrolysis of fd DNA produced smears but no distinct oligonucleotide bands on gel electrophoresis on 6-12% gels, even though a large number of different conditions for hydrolysis were investigated. Conditions aimed at changing the secondary structure of the DNA (1 M urea, 5% formamide, 0.04% SDS, spermidine and putrescine at 0.01 - 0.001 M) inhibited the enzyme but yielded no distinct oligomer bands. By contrast, treatment of MS2 RNA with S1 gave, at optimal conditions of 0.25 M NaCl, 0.02 M Na Acetate pH 4.3 and 0.001 M Zn, a series of pronounced bands at E = 3. After phenol extraction and precipitation with 1 vol. of t BuOH, 70% of the RNA was precipitated. Similarly with P-labeled MS2 RNA, strip chart counting of 2.5% acrylamide - 0.5% agarose electrophoresis patterns of digests of E = 2, 6 or 18 gave count recoveries of 91%, 86%, and 80% respectively, in the form of defined oligonucleotide bands with mobilities less than that of tRNA. Among the latter, four oligomer bands were present in significantly increased amounts. With *Asp. oryzae* (strain 2645, Institute of Applied Microbiology, Japan) various preparations of DNases K_1 and K_2 were obtained. Work is in progress on the purification of both DNases, especially to increase the yields and stability of the enzymes on storage.

Significance to Biomedical Research and the Program of the Institute:
As described in the last annual report, coat-modifications of whole MS2 virus followed by nuclease hydrolysis gave good yields of large oligonucleotides. However, coat-modification did not lower the number of large oligomers obtained, as deduced from comparable hydrolyses of free RNA itself. Such a reduction in the number with a comcomitant increase in amount of large oligomers was sought since in partial hydrolyzates these compounds occur in vanishingly small amounts and are difficult to separate on a preparative scale. For this reason, the single-strand. specific nuclease S_1 of *Asp. oryzae* was tested as a tool for removal of single-stranded portions of *E. coli* phage nucleic acids. With MS2, a reduced number of oligomers is so obtained. The procedures employed to date will be scaled up to obtain mg amounts of large oligomers for sequencing. Availability of the latter compounds, especially if present in pure form, will allow a number of physical and biological studies.

Honors and Awards: None

Publications

Rushizky, G. W., and Mozejko, Jan Henryk,: Preparation of large oligonucleotides by partial RNase T1 hydrolysis of MS2 virus treated with succinic anhydride. Preparative Biochemistry, 3, 425- 437,(1971).

Serial No. NIAMDD-LNE-11
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Protein-nucleic acid interaction: Chromatin structure and restriction in eukaryotic cells.

Previous Serial Number: SAME

Principal Investigator: Dr. Robert T. Simpson

Other Investigators: Dr. Ronald Seale and Miss Linda Propst

Cooperating Units: None

Man Years

Total:	1.0
Professional:	0.5
Other:	0.5

Project Description

Objectives: To study the structural and enzymatic bases for the restriction of translation of the major portion of genetic information in nucleated, mammalian cells; and to study the mechanisms which allow transcription of that portion of the genetic message which is appropriate to the function of the particular cell type.

Methods Employed: Cells in tissue culture (HeLa, a cloned myoblast line and S-91 melanoma cells) are grown in the presence of 5-deoxybromouridine or 5-deoxyiodouridine with or without 5-fluorouridine to substitute part or all of the thymidine residues of DNA with an analogue. Using established methodology, nuclei are isolated from mammalian cells, and then chromatin, the diffuse interphase form of the chromosomes, is isolated from these nuclei. The physicochemical properties of the isolated chromatin are studied using standard biochemical techniques, such as selective chemical modification, circular dichroism, absorption spectrophotometry, ultracentrifugation, hydrogen-ion titration, and interaction with reporter compounds.

Major Findings: The rationale for using acetylation of whole chromatin followed by isolation and characterization of the modified histones as a method of examining DNA-histone interactions in chromatin has been previously presented. Histone fractions isolated by hydroxylapatite chromatography of SDS complexes proved to be contaminated and therefore unsuitable for sequence studies. Currently, affinity chromatograpy on a mercurial-substituted Sepharose is being used to isolate histone f3 (the only histone containing a sulfhydryl group) from the acetylated mixture. Once this histone is removed from the complex

mixture, resolution of the remaining four proteins should be possible by preparative polyacrylamide gel electrophoresis. Sufficient amounts of material should be isolatable to allow sequence study, particularly since Dr. Robert W. Hartley of this laboratory has developed a high sensitivity amino acid analysis system using fluorescamine.

In a number of differentiating systems, the thymidine analogue 5-deoxybromouridine (dBrU) selectively inhibits the production of the proteins characteristic of the differentiated state without any significant effect on the growth rate or viability of the cells. This observation suggests an approach to study of the stable differentiated state in eukaryotes, a state characterized by the production of "luxury molecules", i.e., proteins not necessary for cell growth but characterizing the final differentiated state of a given cell type. As a first effort in study of differentiation through this approach, HeLa cells have been grown in the presence of dBrU and 5-deoxyfluorouridine to block endogenous thymidine synthesis. The properties of the resultant dBrU chromatin have been characterized.

When HeLa cells are grown in the presence of dBrU and dFU, bromouracil is incorporated into DNA as it is synthesized, but the cells do not proceed through the cell cycle, being arrested before mitosis. After 22 hours of incubation, approximately 85-90% of the cellular DNA bands with a density of 1.7500 in neutral CsCl, a density characteristic of unifilar substitution of dBrU for thymidine. The process of DNA synthesis in HeLa with dBrU appears similar to that in control cells with the production of Okazaki pieces followed by ligation of these 4-5 S fragments into intermediate 20-30 S fragments and their eventual joining into mature, high molecular weight DNA. The transition from 20-30 S DNA to high molecular weight DNA is slowed in the presence of dBrU, but still, after a full generation, the major portion of the dBrU substituted DNA sediments in a fashion similar to that of control DNA. Studies with parallel incorporation of dBrU or dT and dA provide no evidence for incorporation of intermediates either enriched or depleted in dBrU content.

The composition of dBrU chromatin is similar if not identical to that of control chromatin, both in terms of mass content of histones, nonhistone chromosomal proteins and RNA and in terms of the distribution of the proteins on gel electrophoresis. Additionally, the dissociation of proteins from control and dBrU chormatin was identical in a pair label experiment using various concentrations of pH 5.5 sodium phosphate in 5 M urea as the dissociant. Protein-nucleic acid interactions (at least their stability) do not seem to differ when dBrU substitutes for dT in DNA of HeLa cells.

In contrast, the physical properties of chromatin substituted with dBrU differs from those of control samples. The melting temperature of dBrU chromatin is 2-3° higher than dT chromtin. However, the melting temperature of the two isolated DNA's differ in a similar fashion. Apparently the dBrU containing DNA is stabilized, probably due to the greater electronegativity of Br when compared to the methyl group. Chromatin proteins add an additional, apparently identical, stabilization to the two DNA's. The conformation of DNA in dBrU-chromatin differs from dT-chromatin as judged by circular dichroism (CD) measurements. The long wavelength psoitive ellipticity band seen in dT-chromatin is

reduced in dBrU-chromatin and a new band, at 260 nm, appears. Similar spectra are seen for isolated dBrU-DNA in high salt concentrations. The CD spectrum of chromatin has been interpreted by others as reflecting a combination of equal contributions from B and C DNA conformations. The spectra of dBrU-chromatin are totally inconsistent with such a conformational assignment, but interestingly, can be adequately fit by a linear combination of C and A forms of DNA structure. The possibility that dBrU induces a conformational change in a portion of chromatin DNA is being pursued through other physical measurements. Additionally, characterization of the biological consequences of the nucleotide substitution, especially as regards the presence of short poly-A sequences in hnRNA, is under investigation.

Significance to Bio-Medical Research and the Program of the Institute:
Determination of the structure of the eukaryotic nucleoprotein complex, chromatin, is necessary for understanding the mechanism of gene regulation in higher organisms. When we can describe in detail the binding of histones to DNA in chromatin, the goal of the acetylation experiments, we will be able to generate meaningful models for protein-DNA interactions in this complex. Study of the molecular consequences of dBrU substitution should allow correlation of altered structure and function of chromatin with altered development of a cell in vivo, furthering our efforts to understand the details of gene regulation in normal and diseased cells.

Honors and Awards: NONE

Publications:

Simpson, R. T.: Structure and Function of Chromatin, Advan. Enzymol. (A. Meister, (ed.) pp. 41-108, Wiley Interscience, 1973.

Piatigorsky, J., Zelenka, P., and Simpson, R. T.: Molecular Weight and Subunit Structure of Delta Crystallin from Embryonic Chick Lens Fibers. Exp. Eye Res. (in press) 1974.

Simpson, R. T. and Seale, R. L.: Characterization of Chromatin Extensively Substituted with 5-deoxybromouridine. Biochemistry, (in press), 1974.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Metabolic studies on chromatin: Synthesis, assembly and turnover of chromatin constitutents.

Previous Serial Number: LNE-18

Principal Investigator: Dr. Ronald Seale

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1.0
Professional:	1.0
Other:	0

Project Description:

Objectives; The theme of this program is the study of functional aspects of chromatin from a biological standpoint. Of immediate interest are problems related to the control mechanism by which synthesis of DNA and histone proteins is coordinated.

Methods Employed: The work this year was centered on two separate studies. 1) synthesis of DNA in the presence of cycloheximide, and 2) isolation of histone F1 messenger RNA. In both cases, studies were performed on HeLa cells in suspension culture. The first study utilized amounts of cycloheximide sufficient to block protein synthesis below the level of detectability. The DNA synthesized under these conditions was labeled with isotopic thymidine and chromatin was then prepared. In some experiments, prior to administration of cycloheximide and label, the DNA was density-labeled with BUdR. Analysis of chromatin proteins was performed by gel electrophoresis and radioactivity determination of gel slices. DNA synthesized in the presence of the drug was examined by alkaline sucrose sedimentation and nuclease digestion of isolated chromatin.

Major Findings:

A. Effects of Cycloheximide on Chromatin Biosynthesis.

In this year, studies of the effects produced by cycloheximide on chromatin replication were completed. When sufficient cycloheximide (CH) is added to totally block protein synthesis in HeLa cells, DNA synthesis proceeds at a step-down rate, 20% that of controls. This residual DNA synthesis persists for

60-90 min after which replication ceases. Inhibitory effects on protein and DNA synthesis are rapidly and fully reversible for at least 45 minutes of CH exposure. By several independent criteria, including the demonstration of Okazaki fragments in the discontinuous DNA replicative process, DNA synthesis in the presence of CH was determined to be a continuation of replication rather than an excision and repair mechanism.

The DNA replicative process is defective, however, because fragments with a 21 S modal distribution accummulate and are not converted to high molecular weight DNA. Upon reversal of inhibition, these fragments are eventually incorporated into high molecular weight DNA by a chain elongation mechanism, but his process takes about 3 hours as compared to 40-60 minutes in untreated cells.

Pool chromatin proteins were demonstrated to exist by prelabeling proteins with a brief (5 minute) pulse of labeled amino acid followed by addition of CH after which radioactivity in both histones and nonhistone chromatin proteins increased by as much as 100% during 60 minutes of residual DNA synthesis. Resolution of these proteins on polyacrylamide gels revealed that the low molecular weight histone, f2a1, was preferentially incorporated during CH inhibition. This pool of proteins, although not measured, probably represents about 0.2% of total chromatin proteins.

Since protein synthesis in CH is effectively zero, chromatin, normally consisting of a complex of DNA and protein, should be demonstrably deficient in protein. Using deoxyribonuclease digestion as a relative measure of the amount of protein complexities to DNA, this deficiency was verified. DNA synthesized is digested to a greater degree than control chromatin when the digestion was performed either with DNase I or micrococcal nuclease. During recovery from CH inhibition, this protein deficiency is replenished but the process takes 3 hours for completion.

In the normal case, briefly pulse-labeled DNA is also deficient in protein as measured by nuclease susceptibility. This effect is transient and no differences are demonstrable after 20 minutes.

The implications of this work regarding the process of chromatin biosynthesis are that while syntheses of DNA and protein are concomitant, proteins are added after replicon-sized DNA fragments are completed (about 20 minutes). Cycloheximide prevents synthesis, and thus, the accumulation of these proteins on newly synthesized DNA.

B. Isolation of Messenger RNA for the Lysine-rich Histone F1.

Polysomal RNA is isolated from S-phase, cells synchronized by thymidine or hydroxyurea treatment. After resolution of the major RNA species in 1600 ml 15-30% sucrose gradients by zonal centrifugation, the region comprising the 5-15 S species is pooled. This RNA is then subjected to preparative polyacrylamide gel electrophoresis and a fraction of RNA is obtained which has the predicted size for the f1 message. This RNA is translatable in an *in vitro* cell-free reticulocyte protein synthesizing system into peptides which have amino acid characteristics of the f1 protein.

This RNA fraction contains 30-40% of sequences which contain poly A as determined by poly U-hydroxylapatite or nitrocellulose filter fractionation. Thus, while the RNA fraction so far isolated contains the desired messenger activity, it is at least 40-60% impure. Further attempts at purification are in progress.

Significance to Bio-Medical Research and the Program of the Institute:

Understanding of control mechanisms in eukaryotic cells is of fundamental importance to progress in basic life sciences and ultimately to applications in medicine. To date, no system of control in eukaryotes is understood nearly as well as prokaryotic mechanisms of induction and repression of expression of macromolecules. Histone synthesis is a temporal, induction-repression phenomenon which occurs during the cell cycle of all eukaryotes. This process is linear and unidirectional and is a prerequisite for cell division. Information relevant to this fundamentally important control mechanism would be of value not only in understanding other eukaryotic controls but in analogous processes of gene expression during development and aberrant control mechanisms which may cause diseases.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAMDD-LNE-13
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Fractionation of chromatin and the study of nonhistone proteins.

Previous Serial Number: SAME

Principal Investigator: Dr. Gerald Reeck

Other Investigators: Dr. Robert T. Simpson and Dr. Herbert A. Sober

Man Years

Total:	1.3
Professional:	1.3
Other:	0

Project Description:

Objectives: To characterize the histone and nonhistone proteins of condensed and extended chromatin fractions obtained using ECTHAM-cellulose and to assess the extent of differences between the nonhistone chromatin proteins of normal and tumor cells.

Methods Employed: Chromatin is fractionated on ECTHAM-cellulose. Fractions are analyzed by circular dichroism spectroscopy, thermal denaturation, and protein content. Chromatin proteins are examined by discontinuous polyacrylamide gel electrophoresis in sodium dodecyl sulfate.

Major Findings: Chromatins isolated from rabbit kidney and brain, calf liver, HeLa cells and duck erythrocytes were subjected to chromatography on ECTHAM-cellulose. With the exception of the duck erythrocyte chromatin, samples from all these sources were resolved into high and low melting nucleoprotein species, as we observed previously for rabbit liver and calf thymus chromatins. A diminution in histone F1 is common to the low melting, late eluted fractions obtained by chromatography of each chromatin, which suggests that the depletion in histone F1 is responsible for the physical properties of the late-eluted material. Consideration of the distribution of low melting sequences in ECTHAM-cellulose chromatography has led us to the conclusion that the low melting, extended nucleoprotein segments of intact chromatin are on the order of 1000 base pairs long.

We have compared nonhistone proteins of early- and late-eluted fractions from ECTHAM-cellulose chromatography of rabbit kidney, calf liver, and HeLa cell chromatins. While, in each case, the gel profiles of early and late eluted

samples were rather similar, distinct differences in a few bands were observed. There did not appear to be any common pattern among the chromatins.

Duck erythrocyte chromatin differed in its chromatographic behavior from all other chromatins examined. Analysis of ECTHAM-cellulose fractions revealed no variation in circular dichroism properties, thermal denaturation profiles, and protein content. This contrasts sharply with similar measurements on all other chromatins. We interpret these results to mean that extended regions analogous the F1-depleted fragments observed in other chromatins do not exist in erythrocyte chromatin. Perhaps the lack of such regions is related to the low template capacity for RNA synthesis of this chromatin.

In a separate line of experimentation, the nonhistone chromatin proteins of several transplantable rat hepatomas and renal tumors were compared by gel electrophoresis to the nonhistone chromatin proteins of normal adult liver and kidney cortex. The variation between the hepatomas and normal liver were much smaller than had been claimed by other investigators. The differences in the patterns appeared to be quantitative rather than qualitative. More striking differences were seen between the kidney tumors and normal kidney cortex.

Significance to Bio-Medical Research and the Program of the Institute:
The specific restriction of transcription of the eukaryotic genome is central to normal differentiation and most likely is equally important in those diseases, including cancer, which can be viewed as abnormalities of differentiation. We are continuing our investigation of the structural basis of transcriptional control by fractionating chromatin on columns of ECTHAM-cellulose. The nonhistone chromatin proteins are widely thought to be the agents which dictate which DNA sequences are transcribed in eukaryotes. They may, therefore, be important in neoplastic transformation. We have also begun studies of nonhistone chromatin proteins tumor cells with the intention of isolating proteins which are enriched in tumors.

Honors and Awards: None

Publications:

Simpson, R. T. and Reeck, G. R.: A comparison of the proteins of condensed and extended chromatin fractions of rabbit liver and calf thymus. Biochemistry 12: 3853-3858, 1973.

Reeck, G. R., Simpson, R. T., and Sober, H. A.: The distribution of histones and nonhistone proteins in the ECTHAM-cellulose fractions of chromatin from several tissues. Eur. Jour. Biochem. (in press) 1974.

Serial No. NIAMDD-LNE-14
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Properties of folic acid γ-glutamyl carboxypeptidase.

Previous Serial Number: SAME

Principal Investigator: Dr. Gerald Reeck

Other Investigators: Mr. Howard Bakerman

Cooperating Units: None

Man Years

Total:	1.2
Professional:	0.2
Other:	1.0

Project Description

Objectives: To develop a rapid enzymatic assay for folate conjugase and to isolate a substantial quantity of an appropriate substrate.

Methods Employed: Conjugase activity is assayed by microbiological techniques and DEAE-cellulose thin-layer chromatography of reaction mixtures. Folates are assayed by microbiological techniques. Enzymatic properties of conjugase are studied using the DEAE-cellulose thin-layer chromatographic system. The isolation of folates is accomplished using column chromatographic methods.

Major Findings: The research of the past year continued the two areas of investigation described in the previous project report: development of a thin-layer chromatography system for analysis of the hydrolysis of folates by conjugase, and isolation of the folates of *Pediococcus cerevisiae*.

DEAE-cellulose thin-layer chromatography was used to study the mechanism of hydrolysis of folates from yeast and *P. cerevisiae* by conjugases from chicken liver and pancreas. The pancreatic liver enzymes reduced the folates to diglutamates and monoglutamates, respectively. The occurrence in enzymatic reaction mixtures of folates of mobilities intermediate between the unhydrolyzed material and products of complete hydrolysis depended both upon the substrate and enzyme: only in the hydrolysis of the *P. cerevisiae* folate by the liver enzyme were such intermediates found. These compounds are presumably folates containing 2 and 3 glutamates.

Our studies on the folates of the folate-requiring bacterium, *P. cerevisiae*, have indicated a greater complexity than anticipated. In earlier investigations,

only a single folate was found in heated extracts of *P.cerevisiae* powder. Using a milder extraction procedure, we now find several folates. The bacteria were sonicated in the presence of sodium dodecyl sulfate to break the cells and simultaneously denature any conjugase present. The broken cells were removed by centrifugation and the supernatant applied to a Sephadex G-25 column which separated the folates from high molecular weight material. After precipitation of the dodecyl sulfate by addition of potassium chloride, the folates were subjected to chromatography on DEAE-cellulose. The content of glutamic acid residues in these compounds has not been determined precisely, but from microbiological assays before and after conjugase treatment, it is clear that each folate contains more than 2 glutamates.

Significance to Bio-Medical Research and the Program of the Institute: Differences in biological activity between oligoglutamyl folates, which are the forms found in nature, and monoglutamyl folates, which have been the subject of intensive study in metabolism, are gradually appearing in the literature. Thus, the isolation and study of the oligoglutamyl forms and the investigation of their synthesis and degradation *in vivo* are of considerable importance. A good deal of control in folate metabolism may well exist that has so far been unrecognized. Our studies are designed to contribute to the growing awareness of the oligoglutamyl folates and their role in biological systems.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LNE-15
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structural analysis of the active and inactive portions of the eukaryotic genome.

Previous Serial Number: SAME

Principal Investigator: Dr. Robert Simpson

Other Investigators: Dr. Ione Polacow, Dr. Gerald Reeck and Miss Linda Propst

Cooperating Units: NONE

Man Years

Total:	2.0
Professional:	1.5
Other:	0.5

Project Description:

Objectives: To determine the physico-chemical basis for the functional differences between the transcribable portion and the remainder of the eukaryotic genome.

Methods Employed: Fractionation of chromatin is performed on a weak anion-exchange cellulose, ECTHAM-cellulose, which had been designed specifically for the chromatography of nucleic acids. Fractions are analyzed by quantitative protein determinations, gel electrophoresis, viscosity, circular dichroism spectroscopy, susceptibility to nucleases, binding properties for small molecules, chemical modification and ability to support RNA synthesis by DNA-dependent RNA polymerase of *E. coli*. Further chromatin fractionation is carried out by sedimentation in preparative sucrose gradients.

Major Findings: We have extended our studies of the structural and functional properties of sonicated chromatin fractionated on ECTHAM-cellulose in two major areas, protein-DNA interactions and support of RNA synthesis by DNA-dependent RNA polymerase of *E. coli*.

Previous studies have characterized early-eluted chromatin as condensed or supercoiled in structure while late-eluted chromatin was thought to be extended in a more DNA-like conformation. Since the physical properties of these two types of chromatin differed so strikingly while their histone contents differed less markedly (1.1 gm/gm DNA for early- and 0.75-0.8 gm/gm DNA for

late-eluted), we have investigated the binding of DNA and histones in an effort to ascertain the basis for these differences. DNA availability has been measured using titration with polylysine and susceptibility to digestion by micrococcal nuclease. For both measurements, we confirm the observations of Clark and Felsenfeld that about 50% of the DNA of whole, unfractionated chromatin is available to the probe molecules. When chromatin is fractionated on ECTHAM-cellulose, different results are obtained for the various chromatin fractions by both measurement methods. The early-eluted chromatin has less nuclease susceptible DNA 30%) and titrates with less polylysine than whole chromatin. In contrast, the late-eluted fractions require more polylysine to titrate and have as much as 70% of their DNA in a conformation susceptible to digestion by micrococcal nuclease.

Complementary measurements for the binding of histone lysyl residues to DNA have been obtained in study of the acetylation of early- and late-eluted fractions by acetic anhydride. The degree of modification of histones f2a1 and f2a2 appears similar in the two chromatin fractions. In contrast, histones f2b and f3 are modified to about 150% of early-eluted incorporation in late-eluted chromatin samples. These results suggest that histone-DNA interactions are looser in late-eluted, extended chromatin, with more DNA and more histone basic residues being available to interact with various chemical probes. A model for generation of the extended structure has been proposed. It envisages supercoiled chromatin constrained in this conformation through bridge formation by histones bound at their amino and carboxyl termini to DNA. Extended chromatin is thought to arise by detachment of one end of the histone bridge allowing extension of the supercoiled DNA, making more histone basic groups available for modification, and more of the DNA accessible to chemical and enzymatic reagents.

Other studies of the physical properties of these two chromatin fractions are also in progress. We are attempting to reconstitute histones, nonhistones, and DNA from early- and late-eluted fractions to ascertain the proteins responsible for the altered conformations. The binding of one particular histone in the two types of chromatin, is being studied by specific labeling of the sulfhydryl groups of histone f3 in the two disparate chromatin types and study of the environment of the modified residue by various spectroscopic techniques.

In the past we have had difficulties in assessment of the "template activity" of chromatin fractions obtained from ECTHAM-cellulose. Lately, we have utilized the methodology developed by Hyman and Davidson for separate measurement of the initiation and propagation phases of polymerase activity applied to transcription of whole chromatin by Cedar and Felsenfeld. In agreement with these latter workers, we find that whole calf thymus chromatin has only 5-10% as many polymerase binding sites as protein-free DNA. Early-eluted chromatin fractions have even fewer polymerase binding sites, some 2-4% as many as DNA. In striking contrast, late-eluted ECTHAM-cellulose chromatin fractions have 30-40% as many polymerase binding sites as does protein-free DNA. Further fractionation of these chromatin samples by sucrose gradient sedimentation allows the preparation from a single starting chromatin sample of fractions having 1-2% as many polymerase sites as DNA and fractions having nearly 50%

as many sites. The overall difference of 25-50 fold in frequency of binding sites for RNA polymerase is striking, particularly considering the many similarities in the composition of the two fractions. As noted previously, these two species do differ quite markedly in structure, suggesting that the mechanism for generation of template active and template inactive portions of the genome may reside in differences in the way components of chromatin interact rather than in the presence of different components in the two regions.

These *in vitro* studies define condensed chromatin as being repressed and extended chromatin as being transcribable. Whether these fractions are also repressed and transcribed *in vivo* is under investigation using hybridization methodology.

Significance to Bio-Medical Research and the Program of the Institute:
Probably the most direct approach to understanding the gross basis for transcriptional regulation in eukaryotic cells is the study of the composition and structure of separated repressed and transcribed segments of the genome. We have developed methodology which seems to allow separation of these two types of nucleoprotein and our current studies have established major differences in both their structure and composition. Extension of these studies to detect more subtle differences in structure between the chromatin species, e.g., mode of histone binding, should allow still more detailed insight into the mechanism of transcriptional regulation and its alterations in development or in disease states.

Honors and Awards: NONE

Publications:

Simpson, R. T.: Separation of repressed and transcribable chromatin. In Anfinsen. C. B. and Schechter, A. N. (eds.): Current Topics in Biochemistry, 1973. New York, N. Y., Academic Press, 1974 pp. 135-185.

Simpson, R. T. and Polacow I.: Protein-DNA interactions in condensed and extended chromatin. Biochem. Biophys. Res. Commun. 55: 1078-1084, 1973.

Simpson, R. T.: Distribution of RNA polymerase binding sites in fractionated chromatin. Proc. Nat. Acad. Sci. U.S.A. (in press) 1974.

Serial No. NIAMDD-LNE-16
1. Nutrition and Endocrinology
2. Developmental Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemistry of pituitary glycoprotein hormones and the role of pituitary proteolytic enzymes in their secretion and activation.

Previous Serial Number: LNE-18

Principal Investigators: Dr. Peter G. Condliffe, LNE, NIAMDD
Dr. Nuran Yulug, LNE, NIAMDD

Other Investigators: Dr. J. A. Gladner, LBP, NIAMDD

Cooperating Units: None

Man Years

Total:	2.0
Professional:	2.0
Other:	0

Project Description:

Objectives: During the past decade, the sub-units of the pituitary hormones TSH, LH and FSH have been isolated from human glands and from several species of animal glands. As a result, the peptide sequences are known for the subunits of b-TSH, b-LH, and b-FSH. Partial information from peptide mapping of the human hormones, together with recombination experiments involving their various sub-units, strongly suggest that the structures of h-TSH and h-LH are very close to those of the bovine.

The demonstration in several laboratories that the glycoprotein hormone from the pituitary and also from the placenta, possess 2 sub-units, not linked by disulfide or other covalent bonds, raises several questions about their synthesis and the regulation of their secretion.

1. Are the sub-units, which each have, between 100 and 120 amino acid residues, synthesized separately and then somehow combined to yield the active hormone or are they derived from a single chain pro-hormone as is the case with insulin?

2. If the sub-units are synthesized separately, are there, in fact, two pro-hormones each of which is activated and which then are combined to yield the active hormone?

3. Do the pituitary proteolytic enzymes play a role in the secretion and activation of these hormones?

Methods Employed: Against this background, a detailed investigation of the pituitary proteolytic enzymes has been started. Their role in the conversion of putative pro-hormonal forms of the glycoprotein hormones to the active forms found in the pituitary and the circulation is being studied using bovine, salmon and human glands.

Results: Frozen bovine pituitaries have been processed for extraction and purification of the glycoprotein hormones. 1.5 kg of lyophilized pituitary were obtained from 20 lbs of pituitaries. This material is being used for preparation of purified b-TSH and b-LH.

Anti-bovine TSH serum has been prepared against partly purified b-TSH in a burro. The burro was originally immunized against b-TSH in 1965-66 and was first boosted in 1967-68. The animal was again boosted in 1973 with NIH-TSH-B6. A total of 1.0 liter serum was obtained in 4 successive bleedings. The 4th bleeding has good anti-TSH activity by radioimmunoassay. Control serum taken prior to boosting had little activity.

Both the control serum and the 4th bleeding have been fractionated by chromatography on DEAE-cellulose and CM-cellulose. The IgG fractions are being used for preparation of affinity columns for adsorption of b-TSH from crude extracts. Preliminary experiments are being carried out with labelled b-TSH to test the efficacy of the Sepharose-anti-b-TSH columns.

Preliminary experiments have been carried out on the extraction and assay of pituitary proteases. Using a denatured hemoglobin substrate, crude pituitary extracts have been assayed for proteolytic activity. Fractionation by centrifugation at 100,000 g showed activity in the pellet and in the supernatant. The protease activity at pH 8.5 was not inhibited by DFP suggesting it is catheptic in nature.

Proposed Course of Project: The action of the pituitary proteases on the sub-units of the hormones will be studied. Differences will be looked for in the proteolysis of the isolated sub-units by standard techniques for study of protein degradation (gel electrophoresis, peptide mapping, etc). The effect of proteolysis on the immunochemical properties of the hormones and the disaggregated sub-units will also be assessed.

Isolation of the pure hormones will be undertaken from crude extracts using affinity chromatography and related techniques. It pituitary protease activity can be successfully inhibited, a search will be made for pro-hormonal components of the pituitary. By analogy with work on insulins, such components should react with antisera directed against the hormones already isolated. Most partly purified preparations of b-TSH contain multiple components some of which appear to be of higher molecular weight. During gel filtration of crude TSH fractions on G-100, a small amount of TSH activity is usually unretarded. Also, in past experiments in which b-TSH extracts were rechromatographed on

CM-cellulose, about 10% of the biological activity was [illegible] unadsorbed; rechromatography of this fraction, which was of low potency, showed that it differed in many respects from the highly purified TSH fractions that were isolated. These experiments were carried out before the development of radio-immunoassays by Berson and Yalow. At this time (before 1960) these experiments were interpreted as being partly denatured, or aggregated material or possibly as TSH adsorbed to other proteins. With the evolution of current ideas about pro-hormones, these old experiments suggest that pro-hormonal forms of TSH and other glycoprotein hormones are waiting to be found.

Honors and Awards: NONE

Publications:

1. Condliffe, P. G.: Purification and biochemical characterization of thyrotropic hormone. In Beeson, S. A. and Yalow, R. S. (eds.): Methods in Investigation and Diagnostic Endocrinology. Amsterdam, North Holland Press, 1973, pp. 447-457.

ANNUAL REPORT SUMMARY

LABORATORY OF BIOCHEMISTRY AND METABOLISM

A. Polysaccharides in Morphogenesis. The study on the heat-stable protein inhibitor of the chitin synthetase activating factor from Saccharomyces cerevisiae has been completed. Previous determinations of the molecular weight by gel electrophoresis in sodium dodecyl sulfate were found to be unreliable because of the small size of the protein. Better values were obtained by gel filtration in the presence of guanidine. The molecular weight appears to be 8500, in approximate agreement with ultracentrifugal data. By amino acid analysis, the absence of the sulfur amino acids, of arginine and of tryptophan was confirmed. The ultraviolet spectrum showed a maximum at 275 mμ, as expected from the lack of tryptophan. The inhibitors from S. cerevisiae and from S. carlsbergensis have about the same molecular sizes, but very different charges. Despite this difference, each of them cross-reacts with the activating factor of the other species.

The newly-found effect of Polyoxin D, an inhibitor of chitin synthetase, on yeast cells in vivo was studied further. The antibiotic causes the appearance of two types of abnormal cells, as observed with phase contrast optics: the "exploded pair" and the "refringent pairs." The formers consist of a mother-daughter cell pair which has lysed, expelling the intracellular material through an orifice near the junction of the two cells; the refringent pair consists of two very luminous cells connected by a very thin neck. In both cases the junction between the two cells, i.e. the site of septum formation appears to be especially affected. This conclusion was confirmed by electron microscopy. In no case a primary septum, the structure which consists of chitin, was observed in the aberrant cells. Furthermore, experiments on the incorporation of tritiated glucose into cell wall polysaccharides showed that only the labeling of chitin was strongly inhibited by Polyoxin D. Very high concentrations of the antibiotic had to be used in vivo as compared with those effective on chitin synthetase in vitro. This appears to be caused in part by a very low permeability of the cell for Polyoxin D, because the addition of dimethylsulfoxide to the growth medium greatly enhanced the action of the antibiotic.

The glucose-6 phosphate-dependent form of yeast glycogen synthetase, which had been purified to homogeneity, as judged by disc-gel electrophoresis, was studied further. The purified enzyme showed the same biphasic kinetics with UDP-glucose which had been observed before with crude preparations. Because the purified enzyme was free from glycogen, the latter could be used as a variable substrate and also gave rise to biphasic Lineweaver-Burk plots.

The glucose-6 phosphate-dependent enzyme was converted into the independent form in crude extracts and reconverted into dependent form after partial purification, by incubation with [γ-^{32}P]ATP. The ^{32}P-labeled enzyme was further purified and was found to contain between one and two phosphate groups per each of the 4 subunits. Treatment with a muscle glycogen synthetase phosphate resulted in the release of the labeled phosphate concomitant with a transformation into independent form. This is the first direct proof that the glucose-6 phosphate-independent and dependent forms are interconverted by a phosphorylation-dephosphorylation mechanism in yeast.

Electrophoresis of the synthetase in sodium dodecyl sulfate gels showed the presence of two bands of very close mobility, which corresponded to a molecular weight around 80,000, about one-fourth that of the intact enzyme molecule. The relative proportion of the two bands varied in different batches, suggesting that one of them might be a proteolytic product of the other. This was confirmed by submitting deliberately the enzyme to proteolysis with pure trypsin or subtilisin, whereby the slower band was transformed completely into the faster one. Concomitantly, the enzyme lost almost completely the requirement for glucose-6 phosphate, with very little loss in total activity. These results could be rationalized when it was found that the proteases excised a peptide containing the phosphate group which confers on the synthetase the property of requiring glucose-6 phosphate for activity. Since the molecular weight of the phosphorous-containing peptide was less than 10% of the intact subunit, it could be concluded that the phosphate group was close to one end of the polypeptide chain. [E. Cabib]

B. Hepatic Recognition and Binding of Glycoproteins. The purification, by affinity chromatography, of an hepatic protein which retains the characteristic properties associated with the membranes has been accomplished. The isolated material, which is water soluble, has been identified as a glycoprotein in which 10% of the dry weight consists of sialic acid, galactose, mannose, and glucosamine in a molar ratio of 1:1:2:2. The integrity of the interminal sialic acid residues and the presence of calcium were shown to be absolute requirements for binding. Physical chemical studies indicated a high degree of aggregation in the final, water-soluble preparation.

In the original studies identifying hepatic plasma membranes as the major locus of binding of asialoglycoproteins, it was noted that the binding capacity of the membranes was significantly reduced by brief exposure to phospholipase C. In light of this observation, the possibility arose that membrane phospholipids participated in the binding reaction as an essential part of the membrane-glycoprotein complex. Detailed investigation of this problem has demonstrated that phospholipids are not directly involved in binding and that the phospholipase induced inhibition of binding is a secondary phenomenon arising from the nonspecific disruption of the lipid constituents of the membrane surface.

Studies with freshly prepared hepatocytes isolated from perfused rat liver are in progress in an attempt to provide a model system, in vitro, which is capable of transporting, as well as binding asialoglycoproteins. Techniques have been developed to distinguish between the surface bound and intracellular ^{125}I-asialo-orosomucoid and are being currently used for kinetic experiments designed to evaluate the parameters determining the rate-limiting steps in the uptake, transport and catabolism of the ingested material. [G. Ashwell]

C. Hormone-Dependent Differentiation of Mammary Gland In Vitro. Insulin-like material can be extracted from insulin-Sepharose. This soluble material elicits super-insulin responses from mammary epithelial cells. It stimulates the accumulation of α-aminoisobutyric acid by virgin mouse mammary cells, which are unresponsive to insulin itself. It exerts a greater stimulatory effect on mammary cells from pregnant mice than insulin. It also is more efficacious than insulin as a stimulant of DNA synthesis and the combined activities of glucose-6 phosphate dehydrogenase and phosphogluconate dehydrogenase. Its specific biological activity on these cells is more than five times greater than that of insulin.

Terminal differentiation of some cells appears to require hormone-dependent critical events which can only occur during a limited portion of the G_1 phase of the cell-cycle. As a consequence, certain cells previously arrested in the cell-cycle must undergo proliferation as a necessary route to this portion of G_1. This is characteristic of terminal differentiation of mammary epithelial cells, and possibly other cell-types. [Y. Topper]

Differentiation of mouse mammary epithelial cells in vitro depends on the presence of a glucocorticoid in addition to insulin and prolactin. Hydrocortisone, together with insulin, has been shown to facilitate accumulation of rough endoplasmic reticulum (RER) during a 4 day culture period. In the absence of the steroid, RER accumulation lasts only for about 2 days, and thereafter the RER disappears rapidly. Additional studies now reveal that other cellular components are also subject to a similar stabilizing effect of glucocorticoid in the presence of insulin. For example, insulin increases glucose-6 phosphate dehydrogenase activity by 50-100% during the first 2 days of culture. During day 3 and 4, however, this insulin-stimulated increase disappears rapidly, and the enzyme activity declines to, or below, the control level. Addition of glucocorticoid prevents the decline and maintains the elevated new steady-state level. Other hormones such as estrogen, progesterone, testosterone, and prolactin are ineffective in this respect.

The ability of hydrocortisone to facilitate sustained accumulation of the enzyme is mimicked by spermidine, but not by spermine, putrescine or Mg ion. Spermidine is effective at a concentration as low as 4×10^{-5} M, which is within the physiological range. This effect of spermidine is specific for hydrocortisone since spermidine does not simulate the effect of either insulin or prolactin.

Pulse-chase experiments with [^{3}H]leucine show that glucocorticoid decreases the average turnover rate of various proteins by at least half as compared to an insulin system. The steroid, by itself or in combination with insulin, does not produce any detectable increase in the rate of protein synthesis. The results indicate that one of the important actions of glucocorticoid during the differentiation of mouse mammary epithelium in vitro is to facilitate the enhanced accumulation of various cellular components by slowing down their degradation. A general mechanism for the stabilizing effect of glucocorticoid is proposed.

Stimulation of milk-protein synthesis in mouse mammary epithelium in vitro requires insulin, hydrocortisone and prolactin. The requirement for hydrocortisone can be replaced by spermidine. The possibility that spermidine mediates the effect of glucocorticoid is also supported by the observation that the cellular spermidine concentration increases before the accelerated synthesis of casein and α-lactalbumin. [T. Oka]

D. Differentiation of Lymphoid Cells. Freshly isolated popliteal lymph node as well as spleen cells synthesize and secrete principally IgG together with a relatively small amount of IgM. After incubation in cell culture for 48 hours in the absence of added antigen such IgG production approached zero while IgM production was enhanced about ten-fold. Therefore, the capacity of cells to secrete immunoglobulins after incubation in culture for 72 hours can be used as a convenient measure of the induction of IgM producing cells.

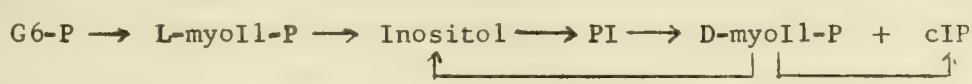
Anti-immunoglobulin antisera specific for light chains and μ heavy chains effectively prevented the induction of immunoglobulin M production. Univalent anti-μ antibody preparations were as inhibitory as bivalent anti-bodies, whereas sera specific for α and γ heavy chains were without effect. These findings suggest that the pertinent cells bear immunoglobulin on their surface before undergoing the induction process.

Anti-rabbit thymocyte antibody can also totally inhibit the induction of IgM production. Univalent as well as bivalent anti-thymocyte antibody preparations were inhibitory when added to cells before the induction of immunoglobulin production had occurred but not afterward. Spleen cells that had been treated with anti-thymocyte antibody and then cultured with thymocytes for 72 hours exhibited an enhanced induction of immunoglobulin production. Untreated spleen cells also showed this property although both untreated lymph node cells and anti-thymocyte antibody treated lymph node cells did not respond to thymocytes. These results indicate that the induction process has an absolute requirement for T (thymus derived) cells, as well as B (bone marrow or bursa derived) cells. Moreover, the enhancement of the induction of immunoglobulin production by lipopolysaccharide, a so-called "thymus independent antigen", was found to be T cell dependent as judged from studies using anti-thymocyte antibody.

Bone marrow cells but not thymus cells, suppress the induction of immunoglobulin production by lymph node cells cultured for 72 hours, in vitro, in the absence of added antigen. Homologous bone marrow cells from immunized as well as unimmunized animals were as inhibitory as autologous bone marrow cells. Unlike the behavior of other inhibitors of the induction process which are inhibitory when added as late as 24 hours after initiation of culture, freshly prepared homologous bone marrow cells were not inhibitory unless added earlier than 24 hours. Heat killed bone marrow cells were as inhibitory as live bone marrow cells, whereas cell-free culture filtrates derived from bone marrow cell cultures were not inhibitory. The inhibitory activity of bone marrow cells was markedly reduced by prior treatment with anti-rabbit immunoglobulin antibody but was unaffected by treatment with anti-sera specific for thymus cells, suggesting that the suppressor cell is some type of B cell although it is incapable of secreting immunoglobulins. [M. Kern]

E. Phospholipid Metabolism in Brain. The large effect of deoxycholate in stimulating the breakdown of endogenous PI to cIP and I1-P reported earlier was confirmed in this laboratory. Although smaller and not consistently reproducible, the effect of acetylcholine was similar, suggesting that breakdown of PI to the observed products is related to PI turnover, which also responds to acetylcholine stimulation.

In addition to the two inositol phosphates a third large peak was observed in stimulated systems. Previously not reported, this compound has been unequivocally identified as glucose-6 phosphate (G6-P). All attempts to correlate its origin with known biosynthetic pathways have failed. Thus the addition of various inhibitors: 2-deoxyglucose (hexokinase), arsenate (phosphorylase), fluoride (mutase), and p-mercuribenzoate, iodoacetate, and iodoacetamide (gluconeogenesis) had no effect on the amount of G6-P produced. Additionally, depletion of glycogen by prolonged brain ischemia did not diminish the compound. G6-P is thus tentatively attributed to the breakdown of an unknown glucose-containing brain phospholipid, possibly similar to those recently described in bacteria. With the finding of stimulated G6-P formation concomitant with PI breakdown, a pathway of PI turnover can be constructed as follows: [F. Eisenberg]



F. Particulate Enzymes of Carbohydrate Metabolism. Microsomal membrane enzyme preparations from homogenates of organs of normal and abnormal rats are obtained by differential centrifugation methods and are activated by treatment with such reagents as deoxycholate, Triton X-100, digitonin or hydroxyl ion. Using these techniques, a liver microsomal enzyme has been found which is capable of catalyzing the formation of glucose-6 phosphate from glucose by the direct transfer of the acid anhydride phosphate group from 1,3-diphosphoglyceric acid without the intermediate formation of adenosine triphosphate.

The enzymes being studied are capable of using PP_i, 1,3-diphosphoglycerate or carbamyl phosphate instead of ATP for the activation of sugars and sugar alcohols prior to their utilization by animal tissues. The quantitative levels of these membrane-bound enzymes have been found to be particularly susceptible in vivo to fasting and to a number of endocrine changes, such as result from diabetes, adrenalectomy, cortisone and insulin administration. Conditions of high glucose levels and increased enzyme activity suggest a physiological function of the PP_i-glucose-phosphotransferase for the synthesis of glucose-6 phosphate in the diabetic animal. [M. Stetten]

G. Synthesis and Degradation of Nucleic Acids. A sensitive assay for measuring a single strand scission of the closed supercoiled molecule of SV40 DNA (nickase activity was developed). This assay depends on two facts: 1) nicked molecules are irreversibly denatured under conditions in which the closed circular supercoiled molecules are not denatured and 2) denatured DNA is specifically hydrolyzed by the S_1 nuclease of A. oryzae. A DEAE-cellulose fractionation of extracts was prepared from nuclei, cytoplasm and whole cells from both infected and noninfected monkey cells. Nickase activity was measured in the different fractions by the S_1 technique and confirmed by ultracentrifugal analysis of products. The existence of nickase activities in nuclear extract or cytoplasmic extract of both infected and noninfected monkey cells was confirmed. The various activities observed in each case, after DEAE-cellulose chromatography were essentially identical, in giving 3 major regions of nickase activity. The first nickase activity eluted from the columns with the wash fluid, and was the major activity. All the characteristics of the activity in this fraction were identical for infected and noninfected cells, and were similar to the nickase activity previously detected in isolated SV40 virions. The second area of nickase activity elucted from the column with low salt, and was similar to the activity in the wash. Again no differences were observed between infected and noninfected cells. The third area of nickase activity eluted from the column with high salt; the activity was minor in amount. The activity was associated with some proteins which were able to interact with SV40 DNA in vitro, and markedly increase the sedimentation rate of the DNA. Thus, the proteins bind to the DNA. These proteins were found exclusively in nuclear extracts. These proteins mimic in some ways the effects of nuclear histones, but our results are not yet conclusive and work is still in progress.

Radioisotope tracer studies confirmed earlier observations by electron microscopy indicating that SV40 DNA synthesis occurs in the nuclei of infected cells. The so-called "M-band" technique was used to initiate studies on the site of SV40 DNA replication in the host nucleus. The results suggest that the majority of the radioactive precursor incorporated into SV40 DNA during short pulses is associated with the host nuclear membrane. The species of SV40 DNA which has been defined as replicative intermediate appears to be associated with the membrane. Preliminary results suggest that mature SV40 DNA is released from the membrane upon completion of replication. In order to study the mechanism of enrichment and replication of defective-substituted SV40, serial undiluted passages were continued in monkey kidney cells. With continuing

passages the percent of host sequences in defective viral DNA increased and at the same time the number of plaque-forming units in the passage yields decreased (4 orders of magnitude). Studies with Hemophilus influenzae restriction endonuclease confirmed earlier findings that the fragments produced by restriction enzyme cleavage are completely different from those obtained with the nondefective viral DNA. Thus, the defective DNA is drastically altered over most of the molecule.

Pulse-label experiments with [^{3}H]thymidine were used to measure rate of DNA synthesis. Upon infection with the yields of later and later passages, the rate of DNA synthesis diminishes to background level. At the 8th passage, the defective virus changed markedly. Suddenly the yield of plaque-forming units increased by about 3 orders of magnitude, the passage gives, upon infection of cells, a rapid cytopathological effect, and rapid viral DNA synthesis. Thus, less defective virus has been produced. Subsequent passaging again leads to more and more defective virus. Highly defective passage yields markedly inhibit the rate of viral DNA synthesis when mixed with nondefective virus prior to infection. This inhibition is dependent on the concentration of infecting defective material and can be overcome by increasing the concentration of nondefective virus.

E. coli A19 (RNase I$^-$) cells were made permeable to nucleotide substrates by toluene treatment. Using these cells with ADP as substrate, poly A was formed and purified by means of poly U-sepharose column chromatography. The size of the poly A was determined by neutral sucrose density gradient centrifugation and HCHO-sucrose gradient centrifugation. Poly A made in these cells was heterogeneous in size ranging from 30S - 16S with a peak at around 25S. Polynucleotide phosphorylase deficient mutants were also used in these studies. No polymerization reaction was detected in these mutant cells but 15% of normal phosphorolysis activity was observed. Using another mutant which produces modified polynucleotide phosphorylase (Q13) no polymerization activity was observed with Mg^{2+} as cofactor and the poly A made with Mn^{2+} as cofactor was smaller in size (12S) than that made in A19 cells. Experiments were designed to detect any RNA species that might contain added poly A sequences. None of the poly A synthesized in the toluenized cells was covalently linked to previously labeled RNA. [M. Singer]

H. Naturally Occurring Sulfur Nucleotides. The first enzyme in the 4-thiouridine(4-TU) thiolation reaction, Factor A, has been purified to homogeneity. It appears that the enzyme can be eluted from cellulose phosphate by a dilute solution of any tRNA or of Poly U, but not by Poly A, Poly C, 3'UMP, UTP, uridine or 4-thiouridine. The apparent binding of the enzyme to Poly U is being investigated, since this polymer is not thiolated in the system.

4-Thiouridine is found naturally only in the No. 8 position in tRNA (except for one known case where a second 4-TU occurs in the 9th spot also). Using pure yeast phenylalanine tRNA, as the sulfur acceptor, the thiolation reaction was carried out in vitro and the labeled product degraded for study. More than half of the sulfur had been incorporated

into a 4-TU in the No. 52 U, in the stem of the thymine loop, and the remainder in the natural No. 8 position.

Spermidine has been reported to facilitate helix formation in Poly U or T, much more so than in other homopolymers. It also binds tightly to the 4-TU region of tRNA. A purified spermidine-tRNA complex was previously found to be unchanged in its competence as a sulfur acceptor, but in this case the final spermidine concentration in the thiolating system was low.

The presence of optimal levels of spermidine (0.01 M) in the thiolating system produces up to 50% decrease in sulfur acceptance by E. coli tRNA. Other tRNAs (yeast, B. subtilis and rabbit liver, as well as E. coli tRNA which has been dethiolated by periodate) show up to a two-fold increase in thiolation rate. All reactions are linear, and preincubation of the enzymes with spermidine before addition of the acceptor tRNA does not change the outcome. Whether this effect is related to the unusually high natural level of preformed 4-TU in E. coli tRNA is being explored. [M.Lipsett]

I. Glutathione Transferases. These studies have continued a two-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups and, as well, on those features which relate to the enzymatic function of proteins.

A group of enzymes, glutathione transferases, present in mammalian livers, catalyze the first step in mercapturic acid formation, the conjugation of glutathione and any of a large number of electrophyllic compounds bearing a group such as halide, nitrite, epoxide and sulfate. Six enzymes have been isolated, five in a homogeneous form and one crystalline which catalyze these reactions. Comparison of these enzymes with a variety of substrates revealed each to be active with more than one class of substrate although there were both qualitative and quantitative restrictions in specificity. As part of a major means of detoxication, these enzymes are being studied with regard to their relationship to each other.

One of these enzymes has been shown to be identical to ligandin the major organic anion binding protein of liver which appears to be the carrier of bilirubin in that organ.

On the level of enzyme chemistry, evidence has been obtained that yeast alcohol dehydrogenase, in the presence of stabilizing concentrations of glycerol, undergoes a reversible conformational change as the result of the presence of the polyol. This change is manifested by a higher affinity for substrate and correlates with changes in the intrinsic fluorescence of the enzyme. [W. Jakoby]

J. Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms. The complex kinetics of unfolding and refolding of globular proteins have previously been interpreted in terms of intermediates on the pathway for unfolding. Conditions have now been found where cytochrome C can unfold and refold by a fast or a slow reaction mechanism. The observations suggest the presence of more than one unfolded state of the protein.

At low pH, pepsinogen rapidly develops proteolytic activity in the presence of high concentrations of denatured hemoglobin. At the same time, it is transformed into a species which is denatured by exposure to pH 8.5, showing that the activity is due to the formation of pepsin, by a unimolecular mechanism, not to an active form of the zymogen. [P. McPhie]

K. The Biochemical Abnormality in Human Cystinosis. Fibroblast cell lines derived from skin biopsies of normal and cystinotic patients are propagated by standard tissue culture methods. Cells originating from cystinotic individuals characteristically possess intracellular levels of cystine (5-15 nanomoles per mg cell protein) many-fold higher than those exhibited by the corresponding cell type from normal individuals (< 0.1 nanomoles per mg protein); such cells thus constitute a convenient source of phenotypic material for various biochemical and metabolic studies. A comparative study of the intracellular amino acid patterns of normal and cystinotic cells failed to reveal any significant qualitative or quantitative difference between the two types of cells. When cystinotic cell lines were incubated at elevated temperatures (40-41°) for 1-4 days a pronounced fall in intracellular cystine levels was observed in comparison with levels exhibited by cells maintained at 37°; cell morphology and viability, as measured by total cell counts, appeared to unaffected by the higher temperature during the incubation period. No change in the usual trace amount of intracellular cystine was noted in normal cells at elevated temperature, nor were changes noted in either normal or abnormal cell lines when incubations were conducted at lower (25°, 31°) temperatures. [F. Tietze]

L. Biochemical Lesions in Genetic Mucopolysaccharidoses. The purification of α-L-iduronidase has been greatly simplified by the use of affinity columns of heparin-agarose and dermatan-agarose which adsorb the enzyme with a high degree of specificity and from which it can be removed by elution with solutions of high ionic strength. A purification of at least 100-fold is achieved in this step.

An assay has been developed for sulfoiduronate sulfatase based on the use of tritium labeled substrate and the separation of product and residual substrate by chromatography or electrophoresis. [I. Leder]

Serial No. NIAMDD-LBM-1
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973, through June 30, 1974

Project Title: The Role of Hepatic Plasma Membranes in the Turnover of Circulating Glycoproteins.

Previous Serial Number: NIAMDD-LBM-1

Principal Investigator: Gilbert Ashwell

Other Investigators: Jean Hickman, Research Chemist
Roger Hudgin, Research Chemist
William Pricer, Research Chemist
Joan Lunney, Post graduate student

Cooperating Units: None

Man Years

Total: 3
Professional: 3
Other:

Project Description:

Objectives:

The major objective of this project has been to investigate the nature of the mechanism whereby hepatic plasma membranes selectively find circulating asialoglycoproteins as a prelude to macromolecular transport and lysosomal catabolism.

Methods Employed:

Hepatic plasma membranes, as well as other intracellular organelles; and intact, viable hepatocytes are isolated by conventional methods of cell fractionation and their binding properties studied with ^{125}I-asialo-orosomucoid as the principal ligand. Solubilization and purification of the binding protein is accomplished by detergent extraction and affinity chromatography.

Major Findings:

The purification, by affinity chromatography, of an hepatic protein which retains the characteristic properties associated with the membranes has been accomplished. The isolated material, which is water soluble, has

Major Findings: (Continued)

been identified as a glycoprotein in which 10% of the dry weight consists of sialic acid, galactose, mannose, and glucosamine in a molar ratio of 1:1:2:2. The integrity of the interminal sialic acid residues and the presence of calcium were shown to be absolute requirements for binding. Physical chemical studies indicated a high degree of aggregation in the final, water-soluble preparation [R. Hudgin and W. Pricer].

In the original studies identifying hepatic plasma membranes as the major locus of binding of asialoglycoproteins, it was noted that the binding capacity of the membranes was significantly reduced by brief exposure to phospholipase C. In light of this observation, the possibility arose that membrane phospholipids participated in the binding reaction as an essential part of the membrane-glycoprotein complex. Detailed investigation of this problem has demonstrated that phospholipids are not directly involved in binding and that the phospholipase induced inhibition of binding is a secondary phenomenon arising from the nonspecific disruption of the lipid constituents of the membrane surface [J. Lunney].

Studies with freshly prepared hepatocytes isolated from perfused rat liver are in progress in an attempt to provide a model system, *in vitro*, which is capable of transporting, as well as binding asialoglycoproteins. Techniques have been developed to distinguish between the surface bound and intracellular ^{125}I-asialo-orosomucoid and are being currently used for kinetic experiments designed to evaluate the parameters determining the rate-limiting steps in the uptake, transport and catabolism of the ingested material [J. Hickman].

Proposed Course of Project:

1. The isolated, water soluble binding protein is a key intermediate for the projected study of the physiological significance of the binding reaction. For this purpose, a high titer antibody has been produced in goats and will be used to monitor the rate and extent of the binding reaction *in vivo*. In addition, the availability of the purified binding protein will provide a means for examining in detail the postulated role for glycosyl transferases in the binding reaction.

2. Studies with intact hepatocytes will be continued in an attempt to clarify the mechanism whereby the binding protein participates in the recognition as well as the uptake and intracellular transport of the ingested material.

Publications:

Ashwell, G. and Morell, A. G.: The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. Advances in Enzymology (1974) (in press).

Publications: (Continued)

Ashwell, G.: The role of cell surface carbohydrates in binding phenonomena. In Jamieson, A. and Robertson, D. M. (eds.): Mammalian Cell Membranes. vol. 4, London, Butterworth (in press).

Hudgin, R. L., Pricer, W. E., Ashwell, G., Stockert, R. J., and Morell, A. G.: The isolation and properties of a rabbit liver binding protein specific for asialoglycoproteins. J. Biol. Chem. (in press).

Pricer, W. E., Hudgin, R. C., Ashwell, G., Stockert, R. J. and Morell, A. G.: A membrane receptor protein for asialoglycoproteins. In Jakoby, W. B. and Wilchek, W. (eds.): Vol. 34, Methods in Enzymology,(in press).

REPORTED IN PRESS - 1973

Ashwell, G.: The role of sialic acid in the immunological and biological properties of glycoprotein hormones. In Jamieson, A. and Robertson, D. M. (eds.): Endocrinology, Proceedings of the Fourth International Congress Washington, D. C. 1972 Amsterdam, Excerpta Medica International Congress Series No. 273, 1972, pp. 601-604.

Serial No. NIAMDD-LBM-2
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Glutathione Transferases

Previous Serial Number: NIAMDD-LBM-2

Principal Investigator: William B. Jakoby

Other Investigators: Michael J. Pabst, Staff Fellow
William H. Habig, Staff Fellow
Joyce S. Myers, Graduate Student
(Hopkins-FAES Program)

Cooperating Units: None

Man Years

Total:	5
Professional:	4
Other:	1

Project Description:

Objectives:

These investigations are concerned with the reactivity of a variety of chemical groupings in enzyme-catalyzed reactions. Choices are such as to lead to investigations of the general phenomena of enzyme mechanisms. Particular attention is presently directed to a series of enzymes forming part of a versatile detoxication system.

Methods Employed:

The approach is at the level of work with isolated purified enzymes and involves their study by chemical and physical methods.

Major Findings:

These studies have continued a two-fold emphasis on the metabolic utilization of a variety of model compounds bearing specific functional groups and, as well, on those features which relate to the enzymatic function of proteins.

Major Findings: (Continued)

A group of enzymes, glutathione transferases, present in mammalian livers, catalyze the first step in mercapturic acid formation, the conjugation of glutathione and any of a large number of electrophyllic compounds bearing a group such as halide, nitrite, epoxide and sulfate. Six enzymes have been isolated, five in a homogeneous form and one crystalline which catalyze these reactions [M. Pabst, W. Habig.] Comparison of these enzymes with a variety of substrates revealed each to be active with more than one class of substrate although there were both qualitative and quantitative restrictions in specificity. As part of a major means of detoxication, these enzymes are being studied with regard to their relationship to each other.

One of these enzymes has been shown to be identical to ligandin [W. Habig, M. Pabst] the major organic anion binding protein of liver which appears to be the carrier of bilirubin in that organ.

On the level of enzyme chemistry, evidence has been obtained that yeast alcohol dehydrogenase, in the presence of stabilizing concentrations of glycerol, undergoes a reversible conformational change as the result of the presence of the polyol [L. Myers]. This change is manifested by a higher affinity for substrate and correlates with changes in the intrinsic fluorescence of the enzyme.

Significance to NIAMDD Research:

Studies of enzyme mechanism and of patterns of metabolism relate directly to the fundamentals of all biological activity. Work on glutathione conjugation by enzymes from liver is intimately related to a general mechanism of aromatic oxidation as well as to the biological problem of detoxication of noxious compounds. The identification of ligandin as a detoxication enzyme provides a new viewpoint to the function of this protein in normal and pathological physiology.

Proposed Course of Project:

It is expected that investigations dealing with the above-noted projects will be continued and a study of other model reactions will be initiated.

Publications:

Fjellstedt, T. A., Allen, R. H., Duncan, B., and Jakoby, W. B.: Enzymatic conjugation of epoxides with glutathione. J. Biol. Chem. 248: 3702-3707, 1973.

Myers, J. S.: The induction of a conformational change in an enzyme by moderate concentrations of glycerol. Ph.D. Dissertation, The Johns Hopkins University, Baltimore, Maryland 1974.

Pabst, M., Habig, W., and Jakoby, W. B.: Mercapturic acid formation: The several glutathione transferases of rat liver. Biochem. Biophys. Res. Commun. 52: 1123-1128, 1973.

Wilchek, M. and Jakoby, W. B.: The literature on affinity chromatography. Methods in Enzymology 34: in press.

Habig, W. H., Pabst, M. J., and Jakoby, W. B.: Glutathione transferases; The first enzymatic step in mercapturic acid formation. J. Biol. Chem. in press.

Pabst, M., Habig, W., and Jakoby, W. B.: Glutathione transferase A: A novel kinetic mechanism in which the reaction pathway depends on substrate concentration. J. Biol. Chem. in press.

Bradbury, S. L., Clark, J. F., Steinman, C. R., and Jakoby, W. B.: Aldehyde dehydrogenase from Baker's yeast. Methods in Enzymology, in press.

Serial No. NIAMDD-LBM-3
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through March 4, 1974

Project Title: Studies on the Synthesis and Degradation of Nucleic Acids

Previous Serial Number: NIAMDD-LBM-3

Principal Investigator: Maxine F. Singer

Other Investigators: Donald LeBlanc, Staff Fellow
G. R. Koteswar Rao, Visiting Fellow
Tikva Vogel, Visiting Fellow

Cooperating Units: Lee Rosner
Laboratory of Molecular Biology
National Institute of Arthritis, Metabolism,
and Digestive Diseases

Man Years

Total: 2.2
Professional: 2.2
Other:

Project Description:

Objectives:

1. To study DNA metabolism in monkey kidney cells infected with Simian Virus 40.

2. To investigate the enzymatic mechanism of the restriction-modification system of coli phage P-1.

3. To study the physiological role of polynucleotide phosphorylase in Escherichia coli.

Methods Employed:

1. Standard tissue culture procedures for growth of monkey kidney cells and Simian Virus 40. Investigation of DNA metabolism utilizes preparative and analytical ultracentrifugation, nucleic acid hybridization, radioisotope tracer technology, and polyacrylamide gel electrophoresis. A variety of chemical procedures are used for fractionation of cellular

Methods Employed: (Continued)

components, DNA, and enzymes. Adaptation of published procedures for the isolation of cell nuclei and nuclear membrane-associated DNA.

2. Genetic methods are used to construct appropriate mutants of coli phage P-1 and lysogens thereof. From these mutants DNA is isolated as substrate, and enzymes are tested for and fractionated in standard ways.

3. E. coli permeable to nucleotide substrates are prepared by toluene treatment and techniques evolved previously for studying polynucleotide phosphorylase reactions were used as was affinity chromatography. Mutants deficient in polynucleotide phosphorylase were used. Density gradient centrifugation provided a means of determining polymer size. Specific degradation of polyribonucleotides was carried out with pancreatic and T_1 ribonucleases.

Major Findings:

1. We developed a sensitive assay for measuring a single strand scission of the closed supercoiled molecule of SV40 DNA (nickase activity). This assay depends on two facts: 1) nicked molecules are irreversibly denatured under conditions in which the closed circular supercoiled molecules are not denatured and 2) denatured DNA is specifically hydrolyzed by the S_1 nuclease of A. oryzae. We then used a DEAE-cellulose fractionation of extracts prepared from nuclei, cytoplasm and whole cells from both infected and noninfected monkey cells. Nickase activity was measured in the different fractions by the S_1 technique and confirmed by ultracentrifugal analysis of products. We confirmed the existence of nickase activities in nuclear extract or cytoplasmic extract of both infected and noninfected monkey cells. The various activities observed in each case, after DEAE-cellulose chromatography were essentially identical, in giving 3 major regions of nickase activity. The first nickase activity eluted from the columns with the wash fluid, and was the major activity. All the characteristics of the activity in this fraction were identical for infected and noninfected cells, and were similar to the nickase activity previously detected in isolated SV40 virions. The second area of nickase activity eluted from the column with low salt, and was similar to the activity in the wash. Again no differences were observed between infected and noninfected cells. The third area of nickase activity eluted from the column with high salt; the activity was minor in amount. The activity was associated with some proteins which were able to interact with SV40 DNA in vitro, and markedly increase the sedimentation rate of the DNA. Thus, the proteins bind to the DNA. These proteins were found exclusively in nuclear extracts. These proteins mimic in some ways the effects of nuclear histones, but our results are not yet conclusive and work is still in progress.

Major Findings: (Continued)

Radioisotope tracer studies confirmed earlier observations by electron microscopy indicating that SV40 DNA synthesis occurs in the nuclei of infected cells. The so-called "M-band" technique was used to initiate studies on the site of SV40 DNA replication in the host nucleus. The results suggest that the majority of the radioactive precursor incorporated into SV40 DNA during short pulses is associated with the host nuclear membrane. The species of SV40 DNA which has been defined as replicative intermediate appears to be associated with the membrane. Preliminary results suggest that mature SV40 DNA is released from the membrane upon completion of replication. In order to study the mechanism of enrichment and replication of defective-substituted SV40, serial undiluted passages were continued in monkey kidney cells. With continuing passages the percent of host sequences in defective viral DNA increased and at the same time the number of plaque-forming units in the passage yields decreased (4 orders of magnitude). Studies with Hemophilus influenzae restriction endonuclease confirmed earlier findings that the fragments produced by restriction enzyme cleavage are completely different from those obtained with the nondefective viral DNA. Thus, the defective DNA is drastically altered over most of the molecule.

Pulse-label experiments with [^{3}H]thymidine were used to measure the rate of DNA synthesis. Upon infection with the yields of later and later passages, the rate of DNA synthesis diminishes to background level. At the 8th passage, the defective virus changed markedly. Suddenly the yield of plaque-forming units increased by about 3 orders of magnitude, the passage gives, upon infection of cells, a rapid cytopathological effect, and rapid viral DNA synthesis. Thus, less defective virus has been produced. Subsequent passaging again leads to more and more defective virus. Highly defective passage yields markedly inhibit the rate of viral DNA synthesis when mixed with nondefective virus prior to infection. This inhibition is dependent on the concentration of infecting defective material and can be overcome by increasing the concentration of nondefective virus.

2. Except for the consistent observation that the two enzymes are extremely difficult to measure reliably in crude extracts or partially purified preparations no significant findings have been made. The difficulties have at least two explanations: 1) the instability of both enzyme activities and 2) interference in the assays by other specific and nonspecific host (E. coli) endonucleases and methylases.

3. E. coli A19 (RNase I$^-$) cells were made permeable to nucleotide substrates by toluene treatment. Using these cells with ADP as substrate, poly A was formed and purified by means of poly U-sepharose column chromatography. The size of the poly A was determined by neutral sucrose density gradient centrifugation and HCHO-sucrose gradient centrifugation. Poly A made in these cells was heterogeneous in size ranging from 30S - 16S with a

Major Findings: (Continued)

peak at around 25S. Polynucleotide phosphorylase deficient mutants were also used in these studies. No polymerization reaction was detected in these mutant cells but 15% of normal phosphorolysis activity was observed. Using another mutant which produces modified polynucleotide phosphorylase (Q13) no polymerization activity was observed with Mg^{2+} as cofactor and the poly A made with Mn^{2+} as cofactor was smaller in size (12S) than that made in A19 cells. Experiments were designed to detect any RNA species that might contain added poly A sequences. None of the poly A synthesized in the toluenized cells was covalently linked to previously labeled RNA.

Significance to NIAMDD Research:

1. Using the methodology and approaches developed and refined in bacterial studies, rigorous studies of nucleic acid metabolism is animal systems are now possible. Studies with viruses are expected to lead to an understanding of the viruses themselves, but also of the nucleic acid reactions in the normal cell. The studies on substituted, defective SV40 have 2 broad implications: First they raise the possibility of transduction experiments in animal systems and Second, the formation of the substituted molecules provides an interesting approach to the reactions accompanying viral infections. In this respect, studying the steps involved in viral DNA replication are of obvious importance.

2. The restriction and modification enzymes have proved of enormous analytical utility in studies of DNA structure and metabolism. Further, restriction and modification (endonuclease and paired methylase) is a major mechanism whereby bacteria protect themselves from infection by bacteriophage and preserve their own DNA. It is widely believed that similar, but as yet unknown processes, are present in mammalian cells.

Proposed Course of Project:

Terminated.

Publications:

Lavi, S., Rozenblatt, S., Singer, M. F., and Winocour, E.: Acquisition of sequences homologous to host DNA by closed circular simian virus 40 DNA. II. Further studies on the serial passage of virus clones. J. Virol. 12: 492-500, 1973.

Rozenblatt, S., Lavi, S., Singer, M. F., and Winocour, E.: Acquisition of sequences homologous to host DNA by closed circular simian virus 40 DNA. III. Host sequences. J. Virol. 12: 501-510 1973.

Serial No. NIAMDD-LBM-4
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Naturally Occurring Sulfur Nucleotides: Identification, Chemistry, Biosynthesis and Biological Significance

Previous Serial Number: NIAMDD-LBM-4

Principal Investigator: Marie N. Lipsett

Other Investigators: Beatrice Chen, Visiting Associate (1 month)

Cooperating Units: None

Man Years

Total:	1.3
Professional:	1.1
Other:	0.2

Project Description:

Objectives:

1. To explore the two-enzyme sulfurtransferase system which forms 4-thiouridine in tRNA.

2. To study the tertiary structure of tRNA as it is reflected in the accessibility of its No. 8 uridine to thiolation.

3. To determine, if possible, the role of 4-thiouridine in the tRNA molecule.

Methods Employed:

Standard separations methods were used for proteins and nucleic acids, i.e., salt fractionation, phase separation, column chromatography and electrophoresis. Sucrose gradients were used for binding studies, along with sephadex separations and filter binding methods.

Major Findings:

1. Purification

The first enzyme in the 4-thiouridine(4-TU) thiolation reaction, Factor A, has been purified to homogeneity. It appears that the enzyme can be eluted from cellulose phosphate by a dilute solution of any tRNA or of Poly U, but not by Poly A, Poly C, 3'UMP, UTP, uridine or 4-thiouridine. The apparent binding of the enzyme to Poly U is being investigated, since this polymer is not thiolated in the system.

2. Sites of *in vitro* thiolation

4-Thiouridine is found naturally only in the No. 8 position in tRNA (except for one known case where a second 4-TU occurs in the 9th spot also). Using pure yeast phenylalanine tRNA, as the sulfur acceptor, the thiolation reaction was carried out *in vitro* and the labeled product degraded for study. More than half of the sulfur had been incorporated into a 4-TU in the No. 52 U, in the stem of the thymine loop, and the remainder in the natural No. 8 position.

3. Differential effects of spermidine

Spermidine has been reported to facilitate helix formation in Poly U or T, much more so than in other homopolymers. It also binds tightly to the 4-TU region of tRNA. A purified spermidine-tRNA complex was previously found to be unchanged in its competence as a sulfur acceptor, but in this case the final spermidine concentration in the thiolating system was low.

The presence of optimal levels of spermidine (0.01 M) in the thiolating system produces up to 50% *decrease* in sulfur acceptance by *E. coli* tRNA. Other tRNAs (yeast, *B. subtilis* and rabbit liver, as well as *E. coli* tRNA which has been dethiolated by periodate) show up to a two-fold *increase* in thiolation rate. All reactions are linear, and preincubation of the enzymes with spermidine before addition of the acceptor tRNA does not change the outcome. Whether this effect is related to the unusually high natural level of preformed 4-TU in *E. coli* tRNA is being explored.

Significance to NIAMDD Research:

The first enzyme in the sulfur-transferase system selects the thiolation site, apparently on the basis of tertiary structure rather than base sequence. Thus this system offers not only a chance of elucidating the role of 4-thiouridine in tRNA, but a chance of using this modified base to study the basic properties of tRNA itself.

Proposed Course of Project:

Studies will be continued on the pure Factor A, including work on the binding reactions with tRNA and Poly U. Further work on the identification of the unknown intermediate which is thiolated by the second enzyme is also planned.

The spermidine effect will be pursued, particularly to see if the presence of spermidine decreases the ambiguity found in the site of thiolation of phenylalanine tRNA. If this turns out to be the case, a detailed comparison of native and thiolated yeast phenylalanine tRNA can be carried out, to try to pick up differences due to the thiolation and thus approach the question of the role of 4-TU.

Publications:

None

Serial No. NIAMDD-LBM-5
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Polysaccharides in Morphogenesis and in the Storage of Energy

Previous Serial Number: NIAMDD-LBM-5

Principal Investigator: Enrico Cabib

Other Investigators: Rodney Ulane, Staff Fellow
James Braatz, Staff Fellow
Angel Duran, Guestworker
Sherwin Kane, Post Doctoral Fellow

Cooperating Units: None

Man Years

Total:	5
Professional:	4
Other:	1

Project Description:

Objectives:

This project attempts to make use of polysaccharide biosynthesis, as a tool in the study of certain fundamental cellular processes, i.e., 1) the mechanisms by which morphological changes are initiated in the course of cell development, as exemplified by the formation of a septum in the yeast cell; 2) the regulatory systems for the biosynthesis of storage material, using glycogen in yeast as a model.

Methods Employed:

Several strains of yeast and their mutants are used as experimental organisms.

Each problem is investigated at the molecular, subcellular and cellular levels. Thus, in addition to the conventional methods of enzymology, gradient centrifugation is used to isolate cell organelles and to determine the intracellular distribution of the different component of the system under study. Furthermore these observations are correlated with others made at the cellular level, using both

Methods Employed: (Continued)

biochemical methods (variations in enzymes and metabolites) and light or electron microscopy (direct observation or autoradiography).

Major Findings:

1. Studies on the intracellular localization of chitin synthetase zymogen in yeast have been pursued further. In order to verify the hypothesis that zymogen is bound to the cytoplasmic membrane, attempts at the isolation of these membranes were continued. One of the problems we faced was how to distinguish the plasmalemma from intracellular membranes in electron micrographs. A possible solution was to obtain fragments of plasmalemma so large, that they could be recognized from their sheer size. The chosen approach was to pretreat briefly intact yeast protoplasts with glutaraldehyde to cross-link the membrane proteins and then lyse the protoplasts in a very alkaline solution. In this way most of the internal organelles are disrupted, but the cytoplasmic membranes, made stronger by the cross-linking, remain very large. The lysate were then fractionated by density-gradient centrifugation and most of the chitin synthetase zymogen was found in the large membrane fraction. Since, however, the increase in specific activity of the enzyme is very small and the membrane fraction is contaminated with other organelles, it will be attempted to reveal directly the localization of the enzyme. Chitin synthesis will be carried out using the membrane fraction as enzyme, with tritiated UDP-N-acetylglucosamine as substrate, and the position of the newly formed polysaccharide will be observed by electron-microscope autoradiography. These experiments are now in progress.

2. The study on the heat-stable protein inhibitior of the chitin synthetase activating factor from Saccharomyces cerevisiae has been completed. Previous determinations of the molecular weight by gel electrophoresis in sodium dodecyl sulfate were found to be unreliable because of the small size of the protein. Better values were obtained by gel filtration in the presence of guanidine. The molecular weight appears to be 8500, in approximate agreement with ultracentrifugal data. By amino acid analysis, the absence of the sulfur amino acids, of arginine and of tryptophan was confirmed. The ultraviolet spectrum showed a maximum at 275 mμ, as expected from the lack of tryptophan. The inhibitors from S. cerevisiae and from S. carlsbergensis have about the same molecular sizes, but very different charges. Despite this difference, each of them cross-reacts with the activating factor of the other species.

3. The newly-found effect of Polyoxin D, an inhibitor of chitin synthetase, on yeast cells in vivo was studied further. The antibiotic causes the appearance of two types of abnormal cells, as observed with phase contrast optics: the "exploded pair" and the "refringent pairs." The formers consist of a mother-daughter cell pair which has lysed,

Major Findings: (Continued)

expelling the intracellular material through an orifice near the junction of the two cells; the refringent pair consists of two very luminous cells connected by a very thin neck. In both cases the junction between the two cells, i.e. the site of septum formation appears to be especially affected. This conclusion was confirmed by electron microscopy. In no case a primary septum, the structure which consists of chitin, was observed in the aberrant cells. Furthermore, experiments on the incorporation of tritiated glucose into cell wall polysaccharides showed that only the labeling of chitin was strongly inhibited by Polyoxin D. Very high concentrations of the antibiotic had to be used in vivo as compared with those effective on chitin synthetase in vitro. This appears to be caused in part by a very low permeability of the cell for Polyoxin D, because the addition of dimethylsulfoxide to the growth medium greatly enhanced the action of the antibiotic.

4. The glucose-6 phosphate-dependent form of yeast glycogen synthetase, which had been purified to homogeneity, as judged by disc-gel electrophoresis, was studied further. The purified enzyme showed the same biphasic kinetics with UDP-glucose which had been observed before with crude preparations. Because the purified enzyme was free from glycogen, the latter could be used as a variable substrate and also gave rise to biphasic Lineweaver-Burk plots.

The glucose-6 phosphate-dependent enzyme was converted into the independent form in crude extracts and reconverted into dependent form after partial purification, by incubation with [γ-^{32}P]ATP. The ^{32}P-labeled enzyme was further purified and was found to contain between one and two phosphate groups per each of the 4 subunits. Treatment with a muscle glycogen synthetase phosphate resulted in the release of the labeled phosphate concomitant with a transformation into independent form. This is the first direct proof that the glucose-6 phosphate-independent and dependent forms are interconverted by a phosphorylation-dephosphorylation mechanism in yeast.

Electrophoresis of the synthetase in sodium dodecyl sulfate gels showed the presence of two bands of very close mobility, which corresponded to a molecular weight around 80,000, about one-fourth that of the intact enzyme molecule. The relative proportion of the two bands varied in different batches, suggesting that one of them might be a proteolytic product of the other. This was confirmed by submitting deliberately the enzyme to proteolysis with pure trypsin or subtilisin, whereby the slower band was transformed completely into the faster one. Concomitantly, the enzyme lost almost completely the requirement for glucose-6 phosphate, with very little loss in total activity. These results could be rationalized when it was found that the proteases excised a peptide containing the phosphate group which confers on the synthetase the property of requiring glucose-6 phosphate for activity. Since the molecular weight of the phosphorous-containing peptide was less than 10% of that of the intact subunit, it could be concluded that the phosphate group was close to one end of the polypeptide chain.

Significance to NIAMDD Research:

The findings related to septum formation in yeast promise to provide for the first time the detailed molecular mechanism for the triggering of a morphological change in the cell. It is hoped that this case will provide a useful model for the general processes of differentiation and cell development.

The regulation of glycogen synthesis, on the other hand, is a model for the problem of energy distribution and storage in the cell. As was remarked in other occasions, there is a striking parallelism between these regulatory mechanisms, as observed in yeast and in mammalian cells. Furthermore, the possibilities of biochemical and genetic experimentation provided by a unicellular organism made of yeast a unique material for these studies.

Proposed Course of Project:

Several aspects of chitin synthesis and septum formation in yeast will be attacked in the future. Many efforts are now dedicated to ascertain the localization of chitin synthetase zymogen in the cell. The purification of the zymogen activating factor by affinity chromatography, using sepharose-bound activating factor inhibitor as the adsorbent, has already begun with encouraging preliminary results. Attempts to obtain temperature-sensitive yeast mutants with lesions in the formation of the septum are also under way.

The availability of a method for the purification of yeast glycogen synthetase in the glucose-6 phosphate-dependent form has made possible to compare the properties of the enzyme from the wild type with that of a mutant, GS1-36, which is unable to accumulate glycogen. It is hoped that some light might be shed in this way on the mutant defect and therefore on the mechanism of regulation of glycogen synthesis in normal cells. Investigations have also begun on the enzymes which interconvert the two forms of glycogen synthetase, a kinase and a phosphatase.

Publications:

Cabib, E., Farkas, V., Ulane, R. E., and Bowers, B.: Yeast septum formation as a model system for morphogenesis. In Villanueva, J. R., Garcia-Acha, I., Gascon, S., and Uruburn, F. (eds.): Yeast, Mould and Plant Protoplasts. New York and London, Academic Press, 1973, pp. 105-116.

Serial No. NIAMDD-LBM-6
1. Biochemistry and Metabolism
2. Enzymes and Cellular Biochemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Thermodynamic and Kinetic Studies of Protein Structure and Enzymic Mechanisms

Previous Serial Number: NIAMDD-LBM-6

Principal Investigator: Peter McPhie

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1
Professional:	1
Other:	

Project Description:

Objectives:

By the use of kinetic techniques, it is hoped to determine the nature of the specificity inherent in the structure of globular proteins. This specificity lies both in the mechanism by which proteins fold from a newly synthetized random chain into exact three dimensional structures and also in the means by which these folded structures behave as enzymes and catalyze biochemical reactions. Both of these aspects are under investigation.

Methods Employed:

Visible and ultraviolet spectroscopy in both manual and recording instruments. Stop flow experiments are performed in an Aminco Morrow apparatus. The Aminco Temperature Jump machine is being converted for operation using ultraviolet optics which are necessary for following many biochemical reactions.

Major Findings:

The complex kinetics of unfolding and refolding of globular proteins have previously been interpreted in terms of intermediates on the pathway for unfolding. Conditions have now been found where cytochrome C can unfold and refold by a fast or a slow reaction mechanism. The observations

Major Findings: (Continued)

suggest the presence of more than one unfolded state of the protein.

At low pH, pepsinogen rapidly develops proteolytic activity in the presence of high concentrations of denatured hemoglobin. At the same time, it is transformed into a species which is denatured by exposure to pH 8.5, showing that the activity is due to the formation of pepsin, by a unimolecular mechanism, not to an active form of the zymogen.

Proposed Course of Project:

Studies on the unfolding and refolding of proteins are being continued. The complexities in the unfolded state, mentioned above, are not apparent by ordinary physical techniques, suggesting that they may be slight. If this is so, it is not clear how they can effect the rate of refolding by a factor of a hundred or more.

The enzyme yeast alcohol dehydrogenase is being studied with regard to its enzymic mechanism, which is much more specific than that of the well known liver enzyme and with regard to its marked instability in solution. Enzymic activity is lost by intramolecular rearrangements, which suggest a marked degree of mobility within the molecule.

Kinetic studies on other enzymes are being performed and are being planned.

Publications:

McPhie, P.: Pepsinogen: Activation by a unimolecular mechanism. Biochem. Biophys. Res. Commun. 56: 789-792, 1974.

Miles, E. W. and McPhie, P.: Studies on the mechanism of action of the β_2 subunit of tryptophan synthetase: Evidence for a rate determining proton abstraction." J. Biol. Chem. 249: 2492-2497, 1974.

Serial No. [illegible]-LBM-7
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Hormone-Dependent Differentiation of Mammary Gland *In Vitro*.

Previous Serial Number: NIAMDD-LBM-7

Principal Investigator: Dr. Yale J. Topper

Other Investigators: Dr. Takami Oka, Dr. Barbara K. Vonderhaar, Dr. Piotr Chomczynski, Dr. Colette S. Freeman

Cooperating Units: None

Man Years:

Total: 5.5
Professional: 4
Other: 1.5

Project Description:

Objectives - To study the molecular and cytological phenomena involved in development of mammary gland.

Methods Employed - Mammary gland explants or mammary epithelial cells are cultured in synthetic media. Synthesis of casein is followed by isolation after the tissue has been pulsed with radioactive precursors. Synthesis of α-lactalbumin is followed by measuring the formation of lactose in tissue extracts. DNA and RNA synthesis are studied using conventional methods. Cell proliferation is determined by cell counts. The accumulation of α-aminoisobutyric acid (AIB) is followed with the use of ^{14}C-AIB or ^{3}H-AIB.

Major Findings - 1. Insulin-like material can be extracted from insulin-Sepharose. This soluble material elicits super-insulin responses from mammary epithelial cells. It stimulates the accumulation of α-aminoisobutyric acid by virgin mouse mammary cells, which are unresponsive to insulin itself. It exerts a greater stimulatory effect on mammary cells from pregnant mice than insulin. It also is more efficacious than insulin as a stimulant of DNA synthesis and the combined activities of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase. Its specific biological activity on these cells is more than five times greater than that of insulin.

2. Terminal differentiation of some cells appears to require hormone-dependent critical events which can only occur during a limited portion of the G_1 phase of the cell-cycle. As a consequence, certain cells previously arrested in the cell-cycle must undergo proliferation as a necessary route

to this portion of G_1. This is characteristic of terminal differentiation of mammary epithelial cells, and possibly other cell-types.

Significance to Bio-medical Research and the Program of the Institute -1. The biological implications of the soluble super-insulin described in this report are not known. Perhaps no physiological counterpart of it exists. Alternatively, it, or an equivalent, may represent the most active form of the hormone, formed by interaction between insulin and target systems in vivo. In either case, information about its chemical structure should contribute significantly to our understanding of the biological activity of insulin.

2. The discovery that, in certain instances, hormones can exert their biological effect only when the target cell is in a particular phase of the cell-cycle offers new opportunities for the study of hormone action. Knowledge of distinctive characteristics of the cell during the responsive phase should facilitate elucidation of the mechanism of the hormone response.

Proposed Course of Project - 1. We will attempt to determine the chemical structure of super-insulin.

2. We will study the nature of the hormone-dependent G_1 events which are necessary for terminal differentiation of mammary epithelial cells.

3. Biochemical effects of prolactin on broken preparations of mammary cells will be investigated.

Honors and Awards: None

Publications:

Vonderhaar, B. K. and Topper, Y. J.: Critical cell proliferation as a pre-requisite for differentiation of mammary epithelial cells. Enzyme 15: 340-348, 1973.

Topper, Y. J. and Vonderhaar, B. K.: Concerning the role of critical cell proliferation in differentiation of mammary epithelial cells. In Cold Spring Harbor Symposium on Control of Proliferation in Animal Cells (in press).

Topper, Y. J., Oka, T. and Vonderhaar, B. K.: Techniques for studying development of normal mammary epithelial cells in organ culture. In Hardman, J. G. and O'Malley, B. W. (Eds): Methods in Enzymology (in press).

Oka, T. and Topper, Y. J.: A soluble super-active form of insulin. Proc. Nat. Acad. Sci. USA (in press).

Serial No. NIAMDD-LBM-8
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Phospholipid Metabolism in Brain

Previous Serial Number: NIAMDD-LBM-8

Principal Investigator: Dr. Frank Eisenberg, Jr.

Other Investigator: Dr. Arun Majumder

Cooperating Units: None

Man Years:

Total: 2
Professional: 2
Other: 0

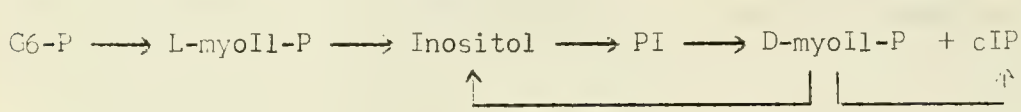
Project Description:

Objectives - An early event after exposure of many cell types to a variety of chemical and physical stimuli is the increased turnover uniquely observed in phosphatidylinositol (PI), a membrane phospholipid. The enzymatic hydrolysis of PI to cyclic inositol 1,2-phosphate (cIP) and inositol 1-phosphate (I1-P) has recently been demonstrated in membranes. The objective of this study is to ascertain whether this breakdown can be correlated with stimulated PI turnover.

Methods Employed - Brain homogenates and particulate fractions were incubated with Ca^{++} and either deoxycholate or acetylcholine in Tris buffer. They were then analyzed for phosphate esters as trimethylsilyl ethers by gas-liquid chromatography in a method developed specifically for this study. In some experiments exogenous PI was added to existing endogenous substrate. In early experiments to establish the identity of compounds observed, peak materials were isolated from the gas stream and characterized chemically and enzymatically.

Major Findings - The large effect of deoxycholate in stimulating the breakdown of endogenous PI to cIP and I1-P reported earlier was confirmed in this laboratory. Although smaller and not consistently reproducible, the effect of acetylcholine was similar, suggesting that breakdown of PI to the observed products is related to PI turnover, which also responds to acetylcholine stimulation.

In addition to the two inositol phosphates a third large peak was observed in stimulated systems. Previously not reported, this compound has been unequivocally identified as glucose 6-phosphate (G6-P). All attempts to correlate its origin with known biosynthetic pathways have failed. Thus the addition of various inhibitors: 2-deoxyglucose (hexokinase), arsenate (phosphorylase), fluoride (mutase), and p-mercuribenzoate, iodoacetate, and iodoacetamide (gluconeogenesis) had no effect on the amount of G6-P produced. Additionally, depletion of glycogen by prolonged brain ischemia did not diminish the compound. G6-P is thus tentatively attributed to the breakdown of an unknown glucose-containing brain phospholipid, possibly similar to those recently described in bacteria. With the finding of stimulated G6-P formation concomitant with PI breakdown, a pathway of PI turnover can be constructed as follows:



Significance to Bio-medical Research and the Program of the Institute - Phospholipids may play a dynamic role in membrane function in addition to their obvious structural role. Since changes in membranes are important in cellular and organellar function, the elucidation of underlying membrane chemistry is essential to the recognition of normal and pathological states.

Proposed Course of Project - Optimal conditions are being sought in an attempt to increase the extent and reproducibility of the acetylcholine effect on PI breakdown. Other neurotransmitters will be tested for their effect in this system. The source of G6-P will be investigated. Studies on the intermediates in the G6-P, L-myoI1-P cyclase reaction will be extended.

Honors and Awards: None

Publications:

Bach, G., Eisenberg, F., Jr., Cantz, M. and Neufeld, E. F.: The defect in the Hunter syndrome: Deficiency of sulfoiduronate sulfatase. Proc. Nat. Acad. Sci. USA 70: 2134-2138, 1973.

Eisenberg, F., Jr.: Gas chromatographic assay of iduronic and glucuronic acid as aldonic acid butaneboronates. Anal. Biochem. (in press).

Serial No. NIAMDD-LBM-9
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Investigations on the Biochemical Abnormality Underlying Human Cystinosis

Previous Serial Number: NIAMDD-LBM-9

Principal Investigator: Dr. Frank Tietze

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives - To determine the underlying biochemical abnormality which leads to the excessive accumulation of the amino acid cystine in certain cells of individuals afflicted with cystinosis, a rare and often fatal inherited disease of young and adult humans.

Methods Employed - Fibroblast cell lines derived from skin biopsies of normal and cystinotic patients are propagated by standard tissue culture methods. Cells originating from cystinotic individuals characteristically possess intracellular levels of cystine (5-15 nanomoles per mg cell protein) many-fold higher than those exhibited by the corresponding cell type from normal individuals (<0.1 nanomoles per mg protein); such cells thus constitute a convenient source of phenotypic material for various biochemical and metabolic studies. Following a variety of experimental manipulations (e.g., changes in medium pH and temperature; labeling with radioactive cystine, etc.) the cells are washed and disrupted under stabilizing conditions and their intracellular complement of low-molecular weight metabolites examined by a number of analytical techniques (e.g., quantitative amino acid analysis, high-voltage electrophoresis, strip counting, etc.).

Major Findings - A comparative study of the intracellular amino acid patterns of normal and cystinotic cells failed to reveal any significant qualitative or quantitative difference between the two types of cells. When cystinotic cell lines were incubated at elevated temperatures (40-41°) for 1-4 days a pronounced fall in intracellular cystine levels was observed in comparison with levels exhibited by cells maintained at 37°; cell morphology

and viability, as measured by total cell counts, appeared to be unaffected by the higher temperature during the incubation period. No change in the usual trace amount of intracellular cystine was noted in normal cells at elevated temperature, nor were changes noted in either normal or abnormal cell lines when incubations were conducted at lower (25°, 31°) temperatures.

Significance to Bio-medical Research and the Program of the Institute - Inasmuch as the excessive levels of cystine in cells of cystinotic patients is known to be compartmentalized within the lysosomal organelles of such cells, cystinosis has been classified as a "lysosomal storage disease". In contrast to other well-studied lysosomal storage diseases, in which metabolites of a polymeric nature accumulate because of the absence or deficiency of lysosomal enzyme activity essential for hydrolytic rupture of specific linkages, cystinosis is characterized by abnormal lysosomal storage of a simple low molecular weight metabolite not possessing any obviously hydrolyzable linkage. Hopefully, a clearer understanding of the specific defect underlying cystine storage may lead to a rational therapeutic approach to arrest the progress of this often fatal disease.

Proposed Course of Project - Normal and cystinotic fibroblasts will be incubated in media containing radioactively labeled (^{35}S; ^{14}C) cystine; after appropriate incubation periods the intracellular contents will be analyzed by electrophoretic technique for differences in labeling patterns of low molecular weight metabolites in order to determine whether some abnormality in the metabolic pathway of cystine exists in the defective cells. Experiments will also continue on the effect of environmental alterations (e.g., changes in pH of the incubation medium) on cystine levels of cystinotic and normal cells. It is further intended to explore the possibility of *in vitro* "correction" of cystinotic cells (i.e., reduction in their cystine content) by the addition of an exogenous source of purified enzymes known to effect the degradative catabolism of cystine.

Honors and Awards: None

Publications:

Tietze, F.: Enzymic reduction of disulfides. In Schulman, J. D. (Ed): Cystinosis. Washington, D. C., U.S. Gov't. Printing Office, 1973, pp. 147-197.

Serial No. NIAMDD-LBM-10
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Role of Spermidine in Hormone-Dependent Differentiation of Mammary Epithelium *In vitro*.

Previous Serial Number: None

Principal Investigator: Dr. Takami Oka

Other Investigator: Mr. John W. Perry

Cooperating Units: None

Man Years:

Total: 1.5
Professional: 1.5
Other: 0

Project Description:

Objectives - To investigate the hormonal regulation of spermidine accumulation and the function of spermidine during development of mammary epithelium.

Methods Employed - The following procedures employed in this investigation are fully described in the list of publications below: organ culture of mouse mammary gland; isolation of epithelial cells; determination of the amount of spermidine by high voltage electrophoresis; enzyme assay for ornithine decarboxylase, S-adenosyl-methionine decarboxylase, arginase, spermidine synthetase and lactose synthetase; determination of the rate of casein, DNA, RNA, and prolactin synthesis.

Major Findings - Differentiation of mouse mammary epithelial cells *in vitro* depends on the presence of a glucocorticoid in addition to insulin and prolactin. Hydrocortisone, together with insulin, has been shown to facilitate accumulation of rough endoplasmic reticulum (RER) during a 4 day culture period. In the absence of the steroid, RER accumulation lasts only for about 2 days, and thereafter the RER disappears rapidly. Additional studies now reveal that other cellular components are also subject to a similar stabilizing effect of glucocorticoid in the presence of insulin. For example, insulin increases glucose-6-phosphate dehydrogenase activity by 50-100% during the first 2 days of culture. During day 3 and 4, however, this insulin-stimulated increase disappears rapidly, and the enzyme activity declines to, or below,

the control level. Addition of glucocorticoid prevents the decline and maintains the elevated new steady-state level. Other hormones such as estrogen, progesterone, testosterone, and prolactin are ineffective in this respect.

The ability of hydrocortisone to facilitate sustained accumulation of the enzyme is mimicked by spermidine, but not by spermine, putrescine or Mg ion . Spermidine is effective at a concentration as low as 4×10^{-5} M, which is within the physiological range. This effect of spermidine is specific for hydrocortisone since spermidine does not simulate the effect of either insulin or prolactin.

Pulse-chase experiments with [^{3}H]leucine show that glucocorticoid decreases the average turnover rate of various proteins by at least half as compared to an insulin system. The steroid, by itself or in combination with insulin, does not produce any detectable increase in the rate of protein synthesis. The results indicate that one of the important actions of glucocorticoid during the differentiation of mouse mammary epithelium in vitro is to facilitate the enhanced accumulation of various cellular components by slowing down their degradation. A general mechanism for the stabilizing effect of glucocorticoid is proposed.

Stimulation of milk-protein synthesis in mouse mammary epithelium in vitro requires insulin, hydrocortisone and prolactin. The requirement for hydrocortisone can be replaced by spermidine. The possibility that spermidine mediates the effect of glucocorticoid is also supported by the observation that the cellular spermidine concentration increases before the accelerated synthesis of casein and α-lactalbumin.

Significance to Bio-medical Research and the Program of the Institute - The polyamine, spermidine, is a naturally occurring polycation and occurs widely in living tissue. Although spermidine has been thought to play an important regulatory role in the processes of growth and differentiation, its physiological function has not been well-defined, at least in mammalian cells. The observations described above may represent the first demonstration of the role of spermidine in cellular function. We intend to pursue further the elucidation of the mechanism of action of spermidine at subcellular and molecular levels in lactogenesis. An understanding of the function of spermidine can serve to reveal the complex regulatory mechanism of cellular development.

Proposed Course of Project - Hormonal interactions on the spermidine turnover in mammary epithelium will be studied. In addition, experiments will be undertaken to elucidate the mechanism of action of spermidine in lactogenesis.

Honors and Awards: None

Publications:

Oka, T.: Effect of glucocorticoid during differentiation of mouse mammary epithelium in vitro. J. Cell Biol. 59, 251a, 1973.

Oka, T.: The role of spermidine in hormone-dependent differentiation of mammary gland in vitro. Science 148, 78-80, 1974.

Oka, T. and Perry, J. W.: Studies on the function of glucocorticoid in mouse mammary epithelial cell differentiation in vitro: Stimulation of glucose-6-phosphate dehydrogenase. J. Biol. Chem. (in press).

Serial No. NIAMDD-LBM-11
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Biochemical Lesions in the Genetic Mucopolysaccharidoses

Previous Serial Number: None

Principal Investigator: Dr. Irwin G. Leder

Other Investigators: None

Cooperating Unit: Section on Human Biochemical Genetics, ARB, NIAMDD

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives - To study the hereditary biochemical defects of lysosome dysfunction which are responsible for a group of interrelated mucopolysaccharidoses and to apply the results of such studies to clinical diagnosis and therapy.

Methods Employed - Two enzymes, α-L-iduronidase and sulfoiduronate sulfatase, are purified from human urine by ammonium sulfate fractionation, molecular sieving and affinity chromatography. The purified proteins are studied for enzymatic activity, corrective factor activity and capture by fibroblasts in tissue culture. Enzyme levels and enzyme capture are studied in cultured fibroblasts from patients and normal individuals.

Major Findings - 1. The purification of α-L-iduronidase has been greatly simplified by the use of affinity columns of heparin-agarose and dermatan-agarose which adsorb the enzyme with a high degree of specificity and from which it can be removed by elution with solutions of high ionic strength. A purification of at least 100-fold is achieved in this step.

2. An assay has been developed for sulfoiduronate sulfatase based on the use of tritium labeled substrate and the separation of product and residual substrate by chromatography or electrophoresis.

Significance to Bio-medical Research and the Program of the Institute - A deficiency of α-L-iduronidase or sulfoiduronate sulfatase results in the Hurler or Hunter syndrome, respectively. The development of techniques for studying these enzymes is useful for diagnostic purposes and their purifi-

cation may make it possible to use them clinically in enzyme replacement therapy.

Proposed Course of Project - 1. Highly specific affinity adsorbants bearing low molecular weight ligands designed to bind the sulfoiduronate sulfatase will be prepared. In addition, a series of lectins will be insolubilized and tested for their ability to bind lysosomal enzymes, not at their catalytic site, but at the site by means of which they are bound to the fibroblast surface.

2. The development of techniques of increased sensitivity for monitoring enzyme levels will be pursued in order to facilitate the assay and study of enzymes and in anticipation of clinical experiments.

Honors and Awards: None

Publications:

Lim, T. W., Leder, I. G., Bach, G. and Neufeld, E. F.: An assay for sulfoiduronate sulfatase (Hunter corrective factor). Carbohydrate Res. (in press).

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Differentiation of Lymphoid Cells

Previous Serial Number: NIAMDD-LBM-12

Principal Investigator: Dr. Milton Kern

Other Investigators: Dr. Daniel H. Zimmerman, Dr. Ko Okumura

Cooperating Units: None

Man Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives - The purpose of this project is to gain insight into the process of differentiation of lymphoid cells with particular emphasis on the cellular conversions and cellular interactions which control the induction of the synthesis and secretion of the major classes of immunoglobulins.

Methods Employed - Lymphoid cells derived from rabbit and mouse were incubated in tissue culture medium and at 24 hour intervals the cells were washed and then incubated again for 90 minutes in leucine-free medium supplemented with [^{3}H]leucine. The resultant secreted [^{3}H]immunoglobulins in the extracellular fluids were analyzed to assess the amount of immunoglobulin M and immunoglobulin G produced. Coprecipitation in the presence of carrier class specific immunoglobulins and class specific antiserums was the fundamental technique used. Molecular sieve chromatography and sodium dodecyl sulfate - acrylamide gel electrophoresis were employed to provide additional verification.

Major Findings - Freshly isolated popliteal lymph node as well as spleen cells synthesize and secrete principally IgG together with a relatively small amount of IgM. After incubation in cell culture for 48 hours in the absence of added antigen such IgG production approached zero while IgM production was enhanced about ten-fold. Therefore, the capacity of cells to secrete immunoglobulins after incubation in culture for 72 hours can be used as a convenient measure of the induction of IgM producing cells.

Anti-immunoglobulin antisera specific for light chains and μ heavy chains effectively prevented the induction of immunoglobulin M production. Univalent anti-μ antibody preparations were as inhibitory as bivalent antibodies, whereas sera specific for α and γ heavy chains were without effect. These findings suggest that the pertinent cells bear immunoglobulin on their surface before undergoing the induction process.

Anti-rabbit thymocyte antibody can also totally inhibit the induction of IgM production. Univalent as well as bivalent anti-thymocyte antibody preparations were inhibitory when added to cells before the induction of immunoglobulin production had occurred but not afterward. Spleen cells that had been treated with anti-thymocyte antibody and then cultured with thymocytes for 72 hours exhibited an enhanced induction of immunoglobulin production. Untreated spleen cells also showed this property although both untreated lymph node cells and anti-thymocyte antibody treated lymph node cells did not respond to thymocytes. These results indicate that the induction process has an absolute requirement for T (thymus derived) cells, as well as B (bone marrow or bursa derived) cells. Moreover, the enhancement of the induction of immunoglobulin production by lipopolysaccharide, a so-called "thymus independent antigen", was found to be T cell dependent as judged from studies using anti-thymocyte antibody.

Bone marrow cells but not thymus cells, suppress the induction of immunoglobulin production by lymph node cells cultured for 72 hours, in vitro, in the absence of added antigen. Homologous bone marrow cells from immunized as well as unimmunized animals were as inhibitory as autologous bone marrow cells. Unlike the behavior of other inhibitors of the induction process which are inhibitory when added as late as 24 hours after initiation of culture, freshly prepared homologous bone marrow cells were not inhibitory unless added earlier than 24 hours. Heat killed bone marrow cells were as inhibitory as live bone marrow cells, whereas cell-free culture filtrates derived from bone marrow cell cultures were not inhibitory. The inhibitory activity of bone marrow cells was markedly reduced by prior treatment with anti-rabbit immunoglobulin antibody but was unaffected by treatment with anti-sera specific for thymus cells, suggesting that the suppressor cell is some type of B cell although it is incapable of secreting immunoglobulins.

Significance to Bio-Medical Research and the Program of the Institute - The abnormal γ-globulins and their subunits that have been observed in serum and urine may be a consequence of aberrations in the secretory mechanism, as well as the synthetic mechanism. Studies from this laboratory have elucidated several new parameters that define synthesis and secretion of normal γ-globulin and provide a means for assessing the similarities and dissimilarities between a normal and abnormal cell.

Arrested differentiation of a cell could lead to the circumstance where such cell multiplies and continues to secrete immunoglobulin of a single specificity in enormous quantities, i.e., as occurs with myeloma. Thus, a

knowledge of lymphoid cell differentiation may well be vital in understanding and perhaps coping with a variety of immunological deficiencies.

Proposed Course of Project - Efforts will be directed toward separating the cell types involved in the induction of immunoglobulin production. Emphasis will be given to a study of requirements for cells and cell-produced mediators.

Honors and Awards: None

Publications:

Zimmerman, D. H. and Kern, M.: Differentiation of lymphoid cells: The in vitro emergence of immunoglobulin M-secreting cells. J. Immunol. 111: 761-769, 1973.

Zimmerman, D. H. and Kern, M.: Differentiation of lymphoid cells: Effect of serum and other mitogenic agents on the selective induction of immunoglobulin M secreting lymph node cells in tissue culture. J. Immunol. 111, 1326-1333, 1973.

Kern, M.: Surface immunoglobulins: Characteristics, mobility and role in immune phenomena. In Jamieson, G. A. and Greenwalt, P. J. (Eds.): Mammalian Cell Membranes, London, Butterworth & Co. (in press).

Okumura, K. and Kern, M.: The absolute requirement for T cells in the induction of IgM secreting cells, in vitro. N. Y. Acad. Sci. (in press).

Serial No. NIAMDD-LBM-13
1. Biochemistry & Metabolism
2. Intermediary Metabolism
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Particulate Enzymes of Carbohydrate Metabolism

Previous Serial Number: NIAMDD-LBM-13

Principal Investigator: Dr. Marjorie R. Stetten

Other Investigator: Mr. Paul Goldsmith

Cooperating Units: None

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Objectives - To study the occurrence, properties and development of membrane bound enzymes of liver and kidney which catalyze the synthesis and hydrolysis of a variety of phosphorylated sugars and sugar alcohols.

Methods Employed - Microsomal membrane enzyme preparations from homogenates of organs of normal and abnormal rats are obtained by differential centrifugation methods and are activated by treatment with such reagents as deoxycholate, Triton X-100, digitonin or hydroxyl ion. Conventional chemical or enzyme assay methods, when applicable, are used for kinetic studies and quantitative measurements. When enzymatic methods are not available, radioactive ^{32}P or ^{14}C incorporation methods are employed. New phosphorylated products are isolated and characterized by chromatographic and chemical methods. Methods for the preparation and isolation of intermediates in the synthesis of lipids and phospholipids are being studied.

Major Findings - A liver microsomal enzyme has been found which is capable of catalyzing the formation of glucose-6-phosphate from glucose by the direct transfer of the acid anhydride phosphate group from 1,3-diphosphoglyceric acid without the intermediate formation of adenosine triphosphate.

Significance to Bio-medical Research and the Program of the Institute - The enzymes being studied are capable of using PP_i, 1,3-diphosphoglycerate or carbamyl phosphate instead of ATP for the activation of sugars and sugar

alcohols prior to their utilization by animal tissues. The quantitative levels of these membrane-bound enzymes have been found to be particularly susceptible *in vivo* to fasting and to a number of endocrine changes, such as result from diabetes, adrenalectomy, cortisone and insulin administration. Conditions of high glucose levels and increased enzyme activity suggest a physiological function of the PP_i-glucose-phosphogransferase for the synthesis of glucose 6-phosphate in the diabetic animal. It is hoped that these studies may contribute to a better understanding of normal and abnormal carbohydrate metabolism.

Proposed Course of Project - The development of glucose-6-phosphatase and related membrane bound enzymes of liver and kidney is being studied in pre- and postnatal rats. In contrast to earlier studies, the enzymes in rough and smooth membrane fractions are being fully activated *in vitro*. The results may help to clarify the early differential development of these membranes.

We have found a liver microsomal membrane enzyme which synthesizes sn-glycerol-1-phosphate, a previously biologically unknown isomer of the common metabolic intermediate, sn-glycerol-3-phosphate. The possible role of sn-glycerol-1-phosphate in the synthesis of fats and phospholipids is being investigated.

Honors and Awards: None

Publications:

Stetten, M. R. and Goldsmith, P. K.: Synthesis of glucose-6-phosphate by a 1,3-diphosphoglycerate-glucose phosphotransferase activity of liver microsomes. Biochim. Biophys. Acta 327: 82-91, 1973.

SECTION ON BIOCHEMICAL MECHANISMS

Chemistry of Fluoroimidazoles

Our discovery of a photochemical route to ring-fluorinated imidazoles resulted in the syntheses, in the first phase of the program, of 2- and 4-fluoroimidazoles, 2- and 4-fluorohistidines, and 2- and 4-fluorohistamines. The widespread biochemical and pharmacological activities of these compounds, as well as their wholly unpredicted chemistry, propelled the initiation of the second phase--preparation and study of a series of more complex fluoroimidazoles. The first of these, 4,5-difluoroimidazole, has now been prepared and, true to its heritage, has opened a new box of chemical and biochemical surprises. Each of the anomalies in chemical behavior has been made the basis of some biochemical application, as described below. (Y. Takeuchi, K. L. Kirk, L. A. Cohen).

Cyclic AMP Phosphodiesterase

The demonstration that imidazole activates this enzyme about 1.5-fold (presumably in a secondary, regulatory site) prompted our exploration of other imidazoles. While 2- or 4-fluoroimidazole proved to have no significant activity, 4,5-difluoroimidazole inhibits the enzyme to a modest degree. Compounds for inhibition of the enzyme are vital to study of the mechanism of action of cyclic AMP and for identification of the various hormones which stimulate its release. The clinical possibilities associated with regulation of the cellular level of cyclic AMP are also considerable. The inhibitors currently in use (caffeine, theophylline) are limited in value because of their poor specificity, high levels required, and their own pharmacological activities. The reasons for the inhibitory activity of 4,5-difluoroimidazole are yet unknown; current studies seek to determine the mechanism of inhibition and the development of more effective imidazole inhibitors (K. L. Kirk, R. Harshman, L. A. Cohen).

2-Fluorohistidine in Protein Biosynthesis and as Receptor Affinity Label

The hydrogen at C-2 in imidazoles is known to undergo facile exchange with D_2O or T_2O at neutral pH, while that at C-4 is essentially inert to exchange. We were surprised to find that H-2, in 4-fluoroimidazoles, is completely resistant to exchange at any pH while H-4, in 2-fluoroimidazoles, is now exchanged readily at pH 10 or above. This lability at H-4 appears to be strongly dependent on the substituent at C-2, since nitrogen, oxygen, or sulfur groups at this position show no such property. This discovery opened the way for a facile preparation of 4-tritio-2-fluorohistidine by simple exchange in T_2O (pH 10). When the labelled amino acid was administered to mice subcutaneously, it entered the main blood pool immediately. After 72 hrs, two-thirds of the total

tritium count was still unexcreted, and half of this amount was found in insoluble protein fractions. Since all of the tritium could be washed out of the amino acid bound into liver protein, the analog must have entered into protein synthesis in place of histidine (i.e., the 2-fluoroimidazole moiety was intact). In brain, however, only 25% of the counts could be exchanged out again. We believe the majority of the amino acid was decarboxylated to 2-fluorohistamine, and that this analog became bound covalently to histamine receptor sites by nucleophilic displacement of the fluorine. Since the fluorine in 2-fluoroimidazoles is known to be reactive toward displacement by protein nucleophiles, and since replacement of the fluorine by another group would render the 4-T nonexchangeable, the explanation given seems the most reasonable for the results. Current studies involve efforts to prove incorporation of the histidine analog in the normal peptide chain, to prove binding to histamine receptor sites in brain, and to determine the fate of the amino acid in the gut. (K. L. Kirk, C. R. Creveling, L. A. Cohen).

Ribonuclease

Ribonuclease-S-peptide has been synthesized with 4-fluorohistidine in place of histidine-12. While the analog combines readily with ribonuclease-S-protein, the adduct shows no enzymatic activity. On the basis of numerous physicochemical and biochemical criteria, the adduct appears to have a conformation very close to that of ribonuclease-S. Most impressive is the fact that substrate analogs protect this adduct against proteolytic digestion to the same degree as ribonuclease-S. It is concluded, therefore, that the fluoroimidazole ring, with a pK 4-5 units lower than that of imidazole-12, is unable to function as a general base in the catalytic process of the enzyme; this observation provides the most conclusive support, to date, for the role assigned to histidine-12 in ribonuclease (B. M. Dunn, C. DiBello, K. L. Kirk, L. A. Cohen, and I. M. Chaiken).

Histidine Ammonia-Lyase

Our earlier work had shown 4-fluorohistidine to be the only effective competitive inhibitor for histidine with this enzyme. Other inhibitor-substrate analogs have now been found: 2-fluorohistidine, 2-aminohistidine, 4-nitrohistidine. Analysis of kinetic data, reversibility, isotope incorporation, and isotope effects prove conclusively that the slow step for this enzyme is not the breakdown of the aminoenzyme, as previously supposed, but the almost concerted loss of a β-hydrogen and the bound amino group. On further study with 4-nitrohistidine, both the metal requirement and the isotope effect are eliminated. It is believed that the rate-determining step has now been advanced to that of formation of the substrate-enzyme complex. This possibility of fixing the rate-determining step of an enzymatic process at one of three different points by small variations in the substrate is unique for enzymology and should provide the basis for new general concepts in the design of inhibitors (C. Klee, P. McPhie, K. L. Kirk, L. A. Cohen).

Urocanase

The product of deamination of histidine by histidine ammonia-lyase, urocanic acid, is converted further by urocanase into a derivative of glutamic acid. This pathway serves as a source of one-carbon units for other biosynthetic schemes. Whether histidine supplies a carbon unit for specific pathways (or for a general pool) and whether it can be replaced by alternative one-carbon sources has yet to be determined. The product of deamination of 2-fluorohistidine, 2-fluorourocanic acid, has been found an extremely potent inhibitor of urocanase. We hope to use this inhibitor as a tool for establishing the significance of the overall degradative pathway in mammalian biosynthesis (C. Klee, K. L. Kirk, L. A. Cohen).

Antibiotic Properties of Fluoroheterocycles

2-Fluorohistidine has been found to inhibit the growth of E. coli (wild) by essentially 100%; 4-fluorohistidine, on the other hand, only delays growth for 24 hrs. Total inhibition is also observed with 2-aminohistidine and delayed growth with 2-fluorothiazoles (analogs of thiamin biosynthesis precursors). The mechanisms of these inhibitions and, presumably, adaptive processes are being investigated (A. Furano, K. L. Kirk, L. A. Cohen).

Fluoro Analogs of Nucleotide Precursors

4-Fluoroimidazole-5-carboxamide ribotide has been synthesized. Preliminary screening shows this compound to be an effective antiviral agent in several systems and moderately effective as an antitumor agent. Studies are in progress for the design and synthesis of more effective analogs and for the preparation of polymers of this compound as possible interferon inducers (J. Reepmeyer, P. Torrence, E. De Clercq, K. L. Kirk, and L. A. Cohen).

Labelling of Biological Materials with Carrier-free ^{125}Iodine

Continuation of our studies in radioiodination has led to the finding that a two-phase process--substrate in neutral, aqueous buffer and I_2 in chloroform or cyclohexane--may be one of the better ways of effecting radioiodine labelling. The reaction is presumed to occur at the liquid-liquid interface; this method appears not only to enhance the reactivity of I_2 but to permit a level of control in degree and site of iodination heretofor unattainable for many cases. The method is currently being tested in a number of laboratories at NIH (R. Jerussi and L. A. Cohen).

Polymer-bound Drugs and Inhibitors

The initial aim of this work was to develop a method for covalent attachment of drugs and inhibitors to a water-soluble polymer, the latter being non-antigenic, compatible with blood, and resistant to enzymatic degradation. Reaction of tyramines or catecholamines with ethylene-maleic anhydride copolymer produces a water-soluble polymer with the amine bound covalently. However, the drug is released very slowly at neutral pH, the rate of release increasing with reduction in pH. While this result was unanticipated, it may prove very useful in developing slow release forms of drugs as well as systems in which

release of a drug is triggered by an undesirable drop in local pH (K. L. Kirk and L. A. Cohen).

Stereopopulation Control

Studies have continued on the kinetics of cyclization of alkylated homophthalic acids. Three important developments have emerged from the study this past year. (1) A linear correlation can be shown between log k for cyclization and the pK of the general acid catalyst, covering the range from acetic to perchloric acid, a spread of 12 pK units. This range is at least twice as great as that attainable in any previous study of general acid catalysis. (2) Catalysis by general base has also been demonstrated; such catalysis is possible only if breakdown of a tetrahedral intermediate is rate limiting. For the first time, general catalysis has been used to prove conclusively the identity of the rate-determining step in a carbonyl reaction. (3) Removal of one methyl group from the 'trialkyl lock' reduces the rate enhancement factor only by 50%. This observation eliminates once and for all any arguments that the enormous rate enhancements observed in this laboratory are due to relief of steric compression; in the last case mentioned, steric compression cannot exist. It now appears clear that 'stereopopulation control' is indeed achieved by freezing of rotational isomerism and that it does provide the best model for the enzyme-substrate complex available to date. (P. S. Hillery and L. A. Cohen).

SECTION ON CARBOHYDRATES

Binding Studies on Homogeneous Immunoglobulins

By screening the binding of J 539 and X 24, two antigalactan immunoglobulins (Fab'), with some thirty synthetic carbohydrate derivatives, it has been possible to map the combining region of these proteins to a large extent. It has been found that, rather than a cleft, the combining region comprises what appears to be a surface area, and that the hapten only makes contact along one side of the molecule. This is the first time that such evidence has been obtained, and is not unlike the structural information being obtained by others for the binding of phosphorylcholine by the immunoglobulin McPC 603 (M. E. Jolley, C. P. J. Glaudemans and E. Zissis).

Conformational changes of immunoglobulins on binding with haptens could perhaps be detected by labeling the protein with a fluorescent label followed by measuring the changes in fluorescence upon hapten binding. Both J-539 and X-24 seem to have a sulfhydryl group which is apparently on the Fc portion of the molecule. Attempts are underway to attach a fluorescent moiety to this sulfhydryl group (M. E. Jolley and C. P. J. Glaudemans).

Mercury-containing Ligand for Phasing of Crystalline J-539 Immunoglobulin Fab'

Fab' J-539 has crystallized (with Drs. Potter and Davies' group). A mercurated hapten has been prepared by us, and it binds very well, its K_a being similar to that of galactosyl $\beta(1\rightarrow6)$ galactose (determined by fluorescence titration) (M. E. Jolley and C. P. J. Glaudemans).

Specificity of Homogeneous Antigalactan Immunoglobulins

Dr. C. Schuerch (Syracuse) has applied for financial support in order to synthesize stereoregular polysaccharides of well defined structures related to our galactans. Dr. Schuerch has been active in this field for some time. Thus we will be able to study the question of specificity. We have already shown that β(1→3) galactans may (weakly) bind to some of our immunoglobulins.

N-acetylhexosaminyl-rhamnose Haptens

Streptococcal polysacchardies are capable of inducing an immune response of restricted heterogeneity in mice. We are presently in the process of synthesizing N-acetylgalactosaminyl 1→3 rhamnose, one of the immunodeterminants involved (E. Zissis and R. K. Ness).

The Preparation of 1-O-unsaturated Esters of Glucuronic Acid

The synthesis of bilirubin diglucuronide has not yet been accomplished. This is so because bilirubin has olefinic double bonds, and the use of glucuronic acid intermediates bearing benzyl blocking groups in the synthesis is prohibited because the removal of these groups (H_2/Pd) after coupling would also reduce the olefinic moiety in bilirubin. Use of O-acetyl blocking groups in the glucuronic acid is also impossible, as their removal would also cleave the ester linkage between the glucuronide and bilirubin. Years ago we researched the possibilities of O-chloroacetate blocking groups in carbohydrate derivatives. These can be removed under neutral conditions (with thiourea in methanol). We have now prepared 2,3,4 tri-O-chloroacetyl D-glucuronic acid in crystalline form. This is a suitable intermediate towards the preparation of 1-O-unsaturated esters of glucuronic acid in general (N. Roy and C. P. J. Glaudemans).

2-Acetamido-2-deoxyaldonic acids

2-Acetamido-2-deoxy-D-gluconic-1,4 lactone--an inhibitor of β-N-acetyl-D-glucosaminidase--has been isolated for the first time in a pure (crystalline) state. This lactone is not as active as the corresponding known 1,5 lactone.

It has been found that both the 2-acetamido-2-deoxy-D-gluconic 1,4 and 1,5 lactones are easily cleaved by alcohols to yield the corresponding esters which also show inhibitory action (M. Pokorny, N. Pravdic', E. Zissis and H. G. Fletcher, Jr.).

Oxidation of N,N diacetylchitobiose to the lactone (of necessity a 1,5 lactone) has yielded a product which also shows inhibitory activity towards β-N-acetyl-D-glucosamidase (R. K. Ness, M. Pokorny and E. Zissis).

Aldonolactones

A biological assay has been employed for the first time in considering the effects that various aldonolactones might have on plant growth. It has

been shown that in the standard Avena coleoptile section test all lactones of aldonic acids (save one) which were tested showed some inhibitory effect. The most active one was cellobiono-1,5-lactone, prepared in crystalline form for the first time in the section. It was shown that the inhibitory activity of these lactones was of a reversible nature and probably involved the blocking of a glycosidase essential for growth (H. Diehl, M. Pokorny, E. Zissis, H. G. Fletcher and R. K. Ness).

Ketoses

Work is in progress on the synthesis of β-fructofuranosyladenine, a potentially cytotoxic substance (R. K. Ness).

SECTION ON MEDICINAL CHEMISTRY

Testing Program - Analgesia, Abuse Liability, Narcotic Antagonist Action

In collaboration with the Committee on Problems of Drug Dependence, NAS, NRC, base-line studies (for analgesia) were made for about 100 new compounds (several prepared by this section) in the hot-plate procedure. About half of these were also tested by the Nilsen method when mixed agonist-antagonist properties were suspected. The physical-dependence and narcotic-antagonist property of many of these were determined at the University of Michigan in the Rhesus monkey.

Two N-cyclopropylmethyl-2-hydroxymorphinans (an optical pair) prepared at Hoffmann-La Roche, Nutley, displayed sufficiently interesting behavior to warrant development as potentially marketable products. Of interest also were two compounds prepared in Medicinal Chemistry. One of these, 6-hydroxy-hexahydrocannabinol showed strong analgesia in the hot-plate and Nilsen procedures, was antagonized by the pure antagonist, naloxone, but appeared to bind to different (in vitro) receptors than narcotic analgesics. Another, N-amylnorketobemidone, 4 times as powerful as morphine displayed no physical dependence liability in a 30-day chronic experiment.

A new solvent system for water insoluble compounds was put to use during the year (L. Atwell, E. L. May).

Acyl Homologs of Heroin

For possible use in "maintenance" programs for "hard-core" heroin addicts, 3,6-diformyl-, 6-formyl-, 3,6-dipropionyl-, and 6-propionyl-morphine were synthesized and stability studies (in aqueous solution) done on them. All were very potent analgesics and were equally potent suppressors of morphine abstinence. 5-Propionylmorphine had a relatively long duration of effect and was stable in aqueous solution (A. E. Jacobson and E. L. May).

Theoretical Analyses in Medicinal Chemistry

CNDO (Complete Neglect of Differential Overlap) calculations have shown that there are differences between the minimum energy conformations of those compounds which do and those which do not have affinity for the site at which norepinep rine is taken up in noradrenergic nerve terminals.

An equation has been developed from Hansch analysis of 60 known 6,7-benzomorphans from which a reasonably accurate estimation of analgesic activity can be made (using standard physico-chemical parameters) for theoretically new compounds of this type (A. E. Jacobson).

Improved Synthesis of (-)-5-m-Hydroxyphenyl-2-Methyl-Morphan

The main drawback in the synthesis of this analgesic of commercial interest was the predominance of O-alkylation in the first step of the synthesis. By a studious selection of solvent and carbanion-forming reagent for m-methoxyphenylcyclohexanone the yield of desired C-alkyl compound was vastly improved. Also the teduim and loss involved in converting the commercially available alkylating agent, $ClCH_2CH_2NMe_2 \cdot HCl$, to free base was circumvented. Optimal conditions were excess sodium hydride with dimethylformamide at low temperatures.

In the course of investigating alternate routes to the title compound, 3a-(m-methoxyphenyl)- and 3a-(m-hydroxyphenyl)-octahydroindoles were prepared and found (by C. R. Creveling, Pharmacodynamics) to be potent inhibitors of the uptake of norepinephrine in mouse heart (M. E. Rogers).

Non-Quaternary-Carbon 6,7-Benzomorphans

As compounds of this type with a 9-methyl substituent have not been accessible via standard methods involving 3-methyl-1,2,5,6-tetrahydropyridines as intermediates, long, rather intricate sequences starting from simple benzene derivatives have been devised. Intermediate in these sequences have been diastereomeric α-tetralone derivatives, one of which (the 3,4-cis-isomer) gave (through its α-bromination product) not the 9α-methyl-6,7-benzomorphan but the β-isomer. The other (3,4-trans-isomer), again through the bromo derivative gave instead of the hoped-for 9α-methyl benzomorphan, only aromatization products (1-naphthol derivatives). Extensive nmr studies (A. Jacobson, R. Wilson, T. Oh-ishi and H. Yeh) helped establish the geometry of the α-tetralones for the des-methoxy compounds. In the methoxy series, nmr studies of the bromo derivatives (H. Inoue) was crucial. The configuration of the 9-methyl substituent of the 6,7-benzomorphans was established by nmr and quaternization (with methyl iodide) rate data. 2,9β-dimethyl-6,7-benzomorphans and the 2'-hydroxy relative (the first tertiary-carbon 9-methyl benzomorphans to be synthesized) are codeine-like and morphine-like, respectively, in mouse analgesia (H. Inoue).

Cannabinoid Research

In order to shed light on the mechanism of action (especially in relation to metabolic products) of Δ^8- and Δ^9-tetrahydrocannabinol (THC), 9-nor-Δ^8-THC, 9-nor-Δ^9-THC and 11-methyl-Δ^8-THC have been synthesized In dogs 9-nor-Δ^8-THC and 11-methyl-Δ^8-THC showed typical cannabinoid-like properties in overt behavior and in cardiovascular effects, indicating that metabolism of Δ^8- and Δ^9-THC to the 11-hydroxy derivatives is not necessary for the mediation of cannabinoid-like biologic activity. 11-Methyl-Δ^8-THC and 9-nor-Δ^8-THC, like Δ^8- and Δ^9-THC, gave no analgesic effect in mice which probably means that the above metabolic conversion is necessary for analgesic activity. 11-Methoxy-Δ^8-THC was only slightly effective as an analgesic.

9-Hydroxyhexahydrocannabinol (9-HCC, both equatorial and axial compounds) and the 9-oxo and 9-amino analogs were also synthesized. The 9(e)-hydroxy compound (especially the levo-isomer) was as potent as morphine in analgesic activity and was antagonized (in mice) by naloxone. Yet 9-HCC binds to different in-vitro receptors than morphine. The axial 9-HCC was inactive as was 9-oxohexahydrocannabinol. The 9-amino compound has not yet been tested (R. S. Wilson).

In Vitro Drug-affinity Studies

N-alkylnorketobemidones (C_9 to C_{10}) have been studied for their ability to displace dihydromorphine in (in vitro) CNS material, presumably narcotic receptors. Their receptor affinities were in good correlation with relative analgesic potencies and in their capacities to antagonize the electrically-induced contractions of the guinea-pig ileum. Thus, in this series, interaction with opiate receptors appears to dictate intrinsic activity. Correlation in the phenylmorphan series (where, as with ketobemidones, the non-fixed phenyl group is equatorial) is also fairly good but less consistent in the benzomorphan series (fixed, axial-phenyl orientation) especially with the 5-H and 5-Me substituted compounds. Thus, predictability for analgesic activity by this method appears still to be somewhat limited. Anomalies arise, particularly with agonist-antagonists, although Pert and Snyder (Johns Hopkins) claim to differentiate compounds with these mixed properties from those having only agonist activity. These studies are being continued to include also possible irreversible inhibitors, fluorescent analgesics, specific analgesics with which antibodies can interact and those which can be used for affinity-labeling research (M. E. Rogers, R. S. Wilson, A. E. Jacobson in collaboration with W. Klee, NIMH; C. Pert and S. Snyder, Johns Hopkins; and H. Kosterlitz, Aberdeen, Scotland).

Experimentation with the Nilsen Method for Analgesic Activity

Modification of pre-shock conditions has resulted in a 25% increase in the number of experimentally acceptable mice. Improvements in the design of the voltage stimulator have been made in order to enhance quantitation and precision (L. Getsiv with I. B. Tice, Biomed. Eng. and Instr. Branch).

Synthesis of Fluorescent Benzomorphans for Binding Studies

2-(N-Dansyl-N-methylaminoethyl)-5,9α-dimethyl-2'-hydroxy-6,7-benzomorphan and the corresponding 5,9-α-diethyl compound have been synthesized for receptor-affinity and analgesic studies in order to attempt to locate analgesic receptors (S. Shiotani and A. E. Jacobson).

Research toward Homomorphinans

A key intermediate, 6-methoxy-9-oxo-4a,10-ethanoiminomethano-1,2,3,4,4a, 9,10,10a-octahydrophenanthrene has been synthesized and its ring-junction stereochemistry established as cis (S. Shiotani).

SECTION ON METABOLITES (in part with Section on Pharmacodynamics)

Effect of Histrionicotoxin on The Ionic Conductance Modulator of the Cholinergic Receptor: A Quantitative Analysis of the End-plate Current.

The effect of histrionicotoxin, the toxin isolated from the Colombian frog, *Dendrobates histrionicus*, was investigated on frog (*Rana pipiens*) sartorius muscles using the voltage clamp technique. After 30 minutes of exposure, histrionicotoxin (3.5×10^{-5} M) reduced the amplitude of the end-plate current (EPC) to 74% of control, shortened the half decay time of the EPC and the membrane potential became more negative, leading to hysteresis in the relationship between the G_{EPC} and the membrane potential. When tetanic stimulation (25 Hz for 1 second) was applied in the presence of histrionicotoxin, the first three EPCs were augmented (36%) and the subsequent EPCs progressively decreased to 40% of the first EPC amplitude. This rate of decrease in the EPC amplitude was greater as the membrane potential became more negative. The EPC amplitude recovered about 30 second after cessation of tetanus. Tetanic stimulation under control conditions resulted in an augmentation of the EPC. It is suggested that histrionicotoxin acts on the ionic conductance modulator of the cholinergic receptor in its open conformation, leading to a reduction of the G_{EPC}. The potential-dependent nature of the suppression of G_{EPC} is interpreted as the manifestation of differential binding affinities of the toxin with different conformational states of the receptor-ionic conductance modulator complex (E. X. Albuquerque, K. Kuba and J. Daly).

The Pharmacology of Batrachotoxin. VI. Effects on the Mammalian Motor Nerve Terminal.

Spontaneous miniature end-plate potentials (m.e.p.p.s) were recorded intracellularly in rat diaphragm muscle preparations bathed in calcium-free Ringer's solution containing 0.5 mM Mg^{2+} and 10 mM EGTA for one hour and then exposed to batrachotoxin (BTX). At 37°C, BTX increased m.e.p.p. frequency from 0.83 to about 150 sec^{-1} and partially depolarized the muscle membrane within 15 minutes. At 28°C, BTX also produced membrane depolarization, but increased the spontaneous m.e.p.p. frequency only to 5 sec^{-1}. In the calcium-free Ringer's solution, veratridine increased the spontaneous m.e.p.p. frequency significantly at 37°C, but had little effect on m.e.p.p. frequency at 28°C. Ouabain also increased spontaneous

m.e.p.p. frequency at 37°C and caused only a slight post-synaptic membrane depolarization. High extracellular concentrations of potassium (20 mM) had little or no effect on the spontaneous m.e.p.p. frequency in the calcium-free Ringer's solution at either 28°C or 37°C. The effects of BTX and veratridine were due to an increase in sodium permeability (P_{Na}) as they were antagonized by tetrodotoxin. In phrenic nerve-diaphragm preparations pretreated with 15 mM Mg^{2+}-Ringer's solution at 34°C, BTX abruptly blocked the end-plate potential in 25 minutes, while the nerve action potential remained unaffected for over one hour. When the end-plate potential was blocked the frequency of spontaneous m.e.p.p.s was either normal or increased. It appears that the invasion of the nerve terminal by the action potential is prevented by a certain critical degree of depolarization of the presynaptic membrane that is less than the degree of depolarization required to induce an increase in m.e.p.p. frequency. The drug-elicited increase in P_{Na} appears to be an initial step in excitation-secretion coupling, since at 28°C BTX caused only membrane depolarization while at 37°C BTX caused both membrane depolarization and increased m.e.p.p. frequency. These results are compatible with the proposal that under calcium-free conditions, spontaneous transmitter release depends on an internal calcium pool, presumably located in the presynaptic membrane, which may be mobilized at a critical temperature by drug-elicited increases in internal sodium concentration (S.-E. Jansson, E. X. Albuquerque and J. Daly).

The Pharmacology of Batrachotoxin. VII. A Structure-activity Relationship of Batrachotoxin and its Analogs and the Effects of pH Alteration.

Batrachotoxin (BTX), the 20α-2,4-dimethylpyrrole-3-carboxylate of batrachotoxinin A, and its analogs were studied on rat diaphragm muscles at 37°C. The modifications to the structure of BTX included: 1) complete substitution of the pyrrole moiety; 2) alterations to the substituents on the pyrrole moiety; 3) cleavage of the 3α-9α hemiketal linkage; and 4) quaternization of the BTX molecule. All of the compounds except compound I, batrachotoxinin A, which lacks the 20α substituent, depolarized the post-synaptic membrane and transiently increased the frequency of spontaneous transmitter release to 400-600 sec^{-1}, and finally producing a blockade of the direct and indirect elicited muscle twitches. Only compound I potentiated the muscle twitches. The 50% depolarizing dose (DD50) was determined for all the compounds and BTX but not for compounds I and VI, dihydrobatrachotoxin, which lacks the 3α-9α hemiketal linkage, for these two analogs never depolarized the postsynaptic membrane more than 10-15%. Although qualitatively similar in their effects, the potencies of the other analogs approximated their LD50: BTX, II (the 20α-2,4,5-trimethyl-pyrrole-3-carboxylate of batrachotoxinin A and VII (the quaternary BTX) were the most potent depolarizing and toxic compounds having DD50s of 4.5, 12 and 110 X 10^{-9} M and LD50s of 2, 1, and 20 μg/kg, i.e. in mice; IV, the 20α-4,5-dimethylpyrrole-3-carboxylate of batrachotoxinin A and III, the 20α-2,4-dimethyl-5-acetylpyrrole-3-carboxylate of batrachotoxinin A, were equally toxic in mice (LD50 = 260 and 280 μg/kg, mice, respectively) but 65- and 110-fold less potent than BTX as depolarizing agents. Compound V, the 20α-p-bromobenzoate of batrachotoxinin A, had an LD50 1000 μg/kg in mice compared to that of BTX (2 μg/kg) and its DD50 was 1000 X 10^{-9} M. Compounds VI and I never depolarized the postsynaptic membrane more than 10-15% at concentrations of 5000 X 10^{-9} M and 7200 X 10^{-9} M, respectively. Each

of the derivatives retained their ability to increase sodium permeability, since the increase in spontaneous miniature end-plate potential frequency and membrane depolarization were reversed by tetrodotoxin and by reducing the external sodium concentration. BTX was found to be more effective at alkaline pH (pH 9-10), where it exists almost entirely as the non-ionized form, than the physiological or acidic pH (6.0). It appears that the analogs of BTX act by a mechanism similar to that of the parent compound but that their potency differs and one or the other compound may have a more selective action on the pre- or postsynaptic membrane. For maximal depolarizing activity and toxicity; 1) a substituted pyrrole moiety is necessary at the 20α position of compound I, batrachotoxinin A; 2) the 3α-9α hemiketal linkage must remain intact providing a rigid structure to the steroid nucleus; and 3) quaternization decreases potency by apparently decreasing lipid solubility (J. E. Warnick, E. X. Albuquerque, R. Onur, S.-E. Jansson, J. Daly, T. Tokuyama and B. Witkop).

A Study of the Irreversible Cholinesterase Inhibitor, Diisopropylfluorophosphate, On Time Course of End-plate Currents in Frog Sartorius Muscle

In the sartorius muscle of the frog, diisopropylfluorophosphate (DFP), an irreversible inhibitor of cholinesterase, reduced the amplitude, shortened the time course (50%) and shifted the equilibrium potential of the end-plate current (EPC) to more negative values. Under control conditions, the half decay time (HDT) of the EPC is potential-dependent; in the presence of DFP it became much less so. Removal of the excess DFP by washing the muscles in normal Ringer's resulted in partial recovery of the EPC amplitude and a 2-fold increase in the HDT. When tetanic stimulation was applied (25 Hz) in the presence of DFP and also after washing, the first five EPCs were augmented and subsequent EPCs gradually declined in amplitude by about 60%. After treatment for 30 minutes with pyridine-2-aldoxime methiodide to dephosphorylate the inhibited sites, and washing, the EPC amplitude and the HDT were restored almost to control values. In the presence of DFP the decay of the EPC followed a two-step exponential function; after washing the preparation prior to pyridine-2-aldoxime methiodide treatment it returned to a one-step exponential function similar to control but with a much larger time constant. At the concentrations used, DFP did not affect the membrane potential or spike activity. It is proposed that DFP acts on both cholinesterase and the ionic conductance modulator for the EPC and that the interaction of ACh with its receptor sites has a much faster time course than that of the resultant conformational changes in the ionic conductance modulator (K. Kuba, E. X. Albuquerque and J. Daly).

A Study of the Cholinergic Receptor in the Normal and Chronically Denervated Mammalian Skeletal Muscles: Effects of d-Tubocurarine, Atropine and α-bungarotoxin.

The cholinolytic activities of d-tubocurarine (d-TC), atropine and α-bungarotoxin (BuTX) at post-junctional regions of normal murine diaphragm and at extrajunctional regions of chronically denervated rat soleus muscles were studied with electrophysiological techniques. All three drugs were able to block end-plate potentials of normal muscles as well as acetylcholine sensitivity of denervated muscles. The effects of atropine and d-TC were

fully reversed upon washing with Ringer's solution, while the effects of BuTX were only partially reversed. The reversibility of BuTX was more evident in denervated muscles than at the normal end-plate, but in both cases only a fraction of the normal response could be detected after intensive washing for at least two hours. The blockade of acetylcholine sensitivity of denervated muscles required a concentration of d-TC 5 to 10 times higher than that necessary to block the end-plate potentials of normal muscle. In both preparations, concentrations of atropine 80 to 100 fold higher than d-TC were required to produce the same degree of blockade. BuTX and d-TC were required to produce the same degree of blockade. BuTX and d-TC did not affect the ionic permeabilities of the muscle fiber during an action potential while atropine decreased both Na^+ and K^+ conductances, the magnitude of the effect being dependent on the frequency of stimulation. At the end-plate region d-TC was much more effective than atropine in protecting against the irreversible effect of BuTX. After denervation, however, neither of the two drugs protected against BuTX. It is concluded that the receptors induced by chronic denervation of skeletal muscles are qualitatively similar in their reactivities to cholinolytic agents to those found at end-plate regions of normal muscles, but that slight differences are responsible for quantitative changes in their interaction with different cholinolytic agents. The data further indicate that atropine interacts with the ionic conductance modulator unit associated with the cholinergic receptor, rather than with the receptor itself (A. J. Lapa, E. X. Albuquerque and J. Daly).

Allenic and Acetylenic Spiropiperidine Alkaloids from the Neotropical Frog, Dendrobates Histrionicus

Four analogs of the acetylenic alkaloid, histrionicotoxin ($C_{19}H_{25}NO$) and the allenic alkaloid, isodihydrohistrionicotoxin have been isolated from extracts of skins of the arrow poison frog, *Dendrobates histrionicus* and characterized as neodihydrohistrionicotoxin, tetrahydrohistrionicotoxin, isotetrahydrohistrionicotoxin and octahydrohistrionicotoxin. These spiropiperidine (8-hydroxy-1-azaspiro[5,5]undecane) alkaloids differ only in the degree of unsaturation in the five carbon (position 2) and four carbon (position 7) side chains. A fifth compound, HTX-D, corresponds in empirical formula to a tetrahydrohistrionicotoxin with a 7-(cis-1-butenyl-3-ynyl) side chain, but the major mass spectral fragmentation with loss of C_2H_5O is not characteristic of the histrionicotoxins. Reduction of histrionicotoxin with hydrogen and Lindlar catalyst affords an isomeric dihydrohistrionicotoxin with the terminal acetylene of the 5 carbon side chain reduced, tetrahydrohistrionicotoxin and hexahydrohistrionicotoxins, while reduction with hydrogen and palladium on charcoal affords dodecahydrohistrionicotoxin which is readily methylated to the tertiary amine by methyl iodide. This investigation was carried out in the LC and under a contract arrangement with Prof. T. Tokuyama at the Osaka City University (T. Tokuyama, K. Uenoyama, G. Brown, J. Daly and B. Witkop).

Synthesis of Histrionicotoxin Without Side Chains

Starting with N-hydroxypiperidine, oxidation to the nitron, Grignard reaction with 4,5-dehydroamylmagnesium bromide, re-oxidation to the nitron and thermal

cyclization and equilibration Dr. E. Gössinger obtained 1,7-dioxo-6-azaspiro-[5,5]undecane which on hydrogenolysis liberated the 7-hydroxyl in the correct position. This compound had none of the activity characteristic of histrionicotoxin.

Other Approaches to Histrionicotoxin

A simple access to lactams derived from 1-azaspiro[5,5]undecans. the skeleton of histrionicotoxin, starts with ethyl cyclopentanone-2-carboxylate, alkylation with 5-bromovaleronitril in the 1-position, Schmidt reaction with hydrazoic acid in chloroform containing concentrated sulfuric acid and Dieckmann cyclization to the desired spirolactams (W. Kissing).

Natural Toxins as Tools in Neurophysiology

Snake venoms (bungarotoxin, cobratoxin), frog poisons (histrionicotoxin, batrachotoxin and atelopidtoxin), and fish toxins (tetrodotoxin, saxitoxin) permit selective inhibition of receptors and ion transport mechanisms in electrogenic membranes. Selective ion binding mechanisms are exemplified by the sodium and lithium complexes of antamanide, the naturally occurring cyclic decapeptide from the mushroom *Amanita phalloides* (G. Brown and B. Witkop).

On the Conformation and Synthesis of Diketopiperazines. 3,4-Dehydroproline Anhydride

In collaboration with Dr. I. L. Karle, Naval Research Laboratory, the program on the alkali ion complexes of cyclic and linear peptides is continuing. Gramicidin A crystals, obtained in the Laboratory of Professor Elkan Blout, Harvard University, were used in their ethanolic-aqueous mother liquor for mapping about 8000 diffractions. The evaluation of these data for electron density calculations is in progress. The amino acid 3,4-dehydro-D,L-proline was prepared from pyrrole-2-carboxylic acid and converted to the hydrochloride of the methyl ester with thionyl chloride in methanol. Liberation of the amino group with triethylamine resulted in the spontaneous formation of the diketopiperazine. Crystal structure analysis by X-ray diffraction showed that both halves of the molecule have the same configuration; i.e., the dimerization occurred between two L molecules or two D molecules rather than between an L and a D molecule. The diketopiperazine ring occurs in a folded conformation with a dihedral angle of 139°. Hydrogen atoms on the two C^{α} atoms are axial and cis to each other. The space group is $P4_12_12$ with $a = 5.607 \pm 0.003$ Å, $c = 28.753 \pm 0.009$ Å, and four molecules in the unit cell (Isabella L. Karle, H. C. J. Ottenheym and B. Witkop).

The Isolation and Characterization of γ-L-glutamyl-S-(*trans*-1-propenyl)-L-Cysteine Sulfoxide from Sandal (*Santalum album* L). An Interesting Occurrence of Sulfoxide Diastereoisomers in Nature.

γ-L-Glutamyl-(S-*trans*-1-propenyl)-L-cysteine sulfoxide (*1*) has been isolated from sandal (*Santal album* L.) where it comprises approximately 0.5% of the weight of the dried leaves.

The structure was proved by nmr, ir and c.d. spectroscopy, by acid and enzymatic hydrolyses and by comparison with a sample of *1* previously isolated from onion (*Allium cepa*).

Circular dichroism measurements established that the sulfoxide group in the sandal and onion peptides are of opposite configurations. (R. Kuttan, N. G. Nair and A. N. Radhakrishnan, Wellcome Research Unit, Christian Medical College Hospital, Vellore-4, Tamil Nadu, India; T. F. Spande, H. J. Yeh and B. Witkop).

A New, Practical Synthesis of L-2-Hydroxytryptophan and Its Derivatives.

D,L-2-Hydroxytryptophan has been prepared by three- or four-step syntheses originating with the reaction products from ethyl 2-(*o*-nitrophenyl)-acetate and diethyl methylenemalonate, isatin and ethyl pyruvate or 3-chloromethyleneoxindole and diethyl formamidomalonate. Yields, however, are modest (15-24% overall) and resolution, when the biologically important L-isomer is desired, poses difficulties.

Acidic hydrolysis of 2,3-dihydropyrrolo[2,3-*b*] indoles which are synthesized easily in a one-step oxidation of N-acetyl-L-tryptophan methyl or ethyl esters with N-bromosuccinimide at pH 8.5-9.0 or preferably with *t*-butyl hypochlorite in triethyl-amine-buffered methylene chloride, after sealed-tube hydrolyses (110°) with constant-boiling HCl afforded L-2-hydroxytryptophan in 75% yield after conversion to the free amino acid by a Dowex-1 (acetate form) column in order to remove HCl while hydrolysis with 20% acetic acid at room temperature provided the N-acetyl methyl and ethyl esters in 63% and 26% yields, respectively. (M. Ohno, T. F. Spande and B. Witkop).

Photocyclization of N-Alicyclic Phthalimides:Synthesis of Multicyclic Benzazepine Systems.

N-Alkylated phthalimides generally undergo photocyclization to cyclobutanols in a Norrish type II photoprocess followed by *retro*-transannular ring opening to benzazepinones. When the 4-alkyl substituent has γ-hydrogen with respect to the newly formed ketonic carbonyl (position 5), type II photoelimination occurs with 4-dealkylation.

Irradiation of N-cyclopentylphthalimide in 10 m*M* acetonitrile solution with a 1 kw high-pressure mercury lamp for 2 hr afforded the 3,4-cyclobutanobenzazepinone in 48% yield whose 3,4-*cis*-junction was confirmed by independent synthesis from 3-ethoxyisoindolone and cyclopentene. Likewise, N-cyclohexylphthalimide afforded, as expected, the homologous benzazepinone whose structure was established by direct comparison with an authentic sample. In addition a new compound, mp 244-246°, was isolated whose yield on photolysis in *t*-butanol increased to 23%, while N-cyclohexylphthalimide under the same conditions afforded exclusively another novel product in 55% yield, of which an X-ray diffraction analysis of a single crystal established the complete structure as 1,2,2a,3,4,9b-hexahydro-9b-acetoxy-9b-acetoxy-1,3-propano-5Hcyclobuta-[d][2]benzazepin-5-one. (Y. Kanaoka, K. Koyama; J. L. Flippen, I. L. Karle, and B. Witkop).

Oxidative and Nonoxidative Pathways in the Photocyclization of Five-Membered Heterocyclic Anilides.

Heterocyclic anilides, such as thiophene-2-and indole-2-carboxanilide, undergo photocyclization in the presence of dissolved oxygen to afford the heterocyclic condensed quinolones. Where as *oxidative* cyclizations of anilide systems are closely related to the well-known photoreactions in the stilbene series, Chapman, *et al.* established *nonoxidative* photocyclization of arylenamines.

On irradiation in a mixture of benzene and ethanol (10 :1 v/v; 200 mg/200 ml) with a 100 W high-pressure mercury lamp for 2 hr, thiophene-2-carboxanilide cyclized oxidatively to 3,4(a,b) thiophenoquinolone-2 in 45% yield, accompanied by a crystalline product A with the same composition as the starting material B. When the reaction was performed under a stream of nitrogen in a solvent free of oxygen, A was obtained as the major product (50%) along with a trace of B. Although A was converted into B by iodine oxidation, it is unlikely to be the true intermediate of the transformation because on irradiation under aerobic conditions A afforded B but with much lower efficiency. Thus A must be the product arising from a plausible intermediate, as a result of a process that competes with the direct oxidation to B. The complete structure of A was directly established by X-ray analysis as *trans*-3,4-dihydro-3,4(a,b)thiophenoquinolone-2. (Y. Kanaoka, I. Kazuhiko, J. D. Flippen, I. L. Karle and B. Witkop).

Interferon Induction by Synthetic Polynucleotides: Importance of Purine N-7 and Strandwise Rearrangement.

The antiviral activity and interferon-inducing ability of single-, double-, and triple-stranded polynucleotides, modified at pyrimidine C-5 or purine N-7, were evaluated in primary rabbit kidney cells challenged with vesicular stomatitis virus. (1) There is a parallel increase in antiviral activity and the temperature at which double-stranded polynucleotides rearrange to inactive triple-stranded complexes. (2) When the purine N-7 of $(A)_n$ is replaced by CH, all resulting double-stranded complexes fail to provide antiviral protection or to induce interferon, even though such complexes meet all requirements previously recognized for interferon induction. (3) Competition experiments between inactive and active polynucleotides indicate that single-stranded polynucleotides apparently do not bind to the cellular receptor sites for interferon induction, whereas triple-stranded complexes and inactive double-stranded complexes bind to such receptor sites but, probably for conformational reasons, fail to trigger the necessary message for interferon induction. (E. De Clercq, P. F. Torrence and B. Witkop).

Interferon Induction as a Tool for the Establishment of Interactions Among Homopolyribonucleotides.

Interferon induction in primary rabbit kidney cells is one of the most sensitive probes to determine the strandedness (single, double or triple) of polynucleotides. Use of this assay system in conjunction with standard techniques such as density gradient centrifugation, ribonuclease resistance, mixing curves and melting profiles have led to the establishment of the following previously unrecognized polynucleotide interaction:

a) poly(A)·poly(U) + poly(I) → poly(A)·poly(U)·poly(I)
b) poly(A)·poly(rT) + poly(I) → poly(A)·poly(rT)·poly(I)
c) poly(A)·poly(U) + poly(dUz) → poly(A)·poly(dUz)·poly(U)
d) poly(A)·2 poly(I) + 2 poly(U) → poly(A)·2 poly(U) + 2 poly(I)

Certain of these reactions may occur at the cellular level as well as in the test-tube *in vitro*.

The following interactions are currently under examination:

e) poly(A)·poly(U) + poly(X) →
f) poly(A)·poly(X) + poly(U) →
g) poly(A)·poly(U) + poly(G),poly(G,U),poly(I,U), GpG or G →
h) poly(A)·poly(U)·poly(I) + poly(U) →

Such reactions are important in understanding the thermodynamics of helix-coil interactions and helix stability, secondary and tertiary structure in RNA/or DNA) and possibly mechanisms of regulation of gene expression. (P. F. Torrence, E. De Clercq, W. E. Stewart II, P. De Somer, B. Witkop).

A Novel Potent Interferon Inducer Derived from Poly(7-deazainosinic Acid).

To determine whether or not purine N-7 of poly(I) plays a significant role in the induction of interferon by poly(I)·poly(C), poly(7-deazainosinic acid) [poly(c^7I)] was prepared by the *Micrococcus luteus* polynucleotide phosphorylase-catalysed polymerization of 7-deazainosine 5'-diphosphate, synthesized from 7-deazainosine (4-hydroxy-7(β-D-ribofuranosyl)-7H-pyrrolo[2,3d] pyrimidine). Poly(c^7I) was, like poly(I), degraded to the nucleoside or nucleotide level by T_1 ribonuclease, bovine spleen phosphodiesterase, snake venom phosphodiesterase, micrococcal nuclease and 0.3N KOH but was totally resistant to degradation by pancreatic ribonuclease A. Unlike poly(I), poly(c^7I) showed little temperature-dependent hyperchromicity in 1.0 M NaCl with an indication of structure only below room temperature. Mixing curves as a function of wavelength, isosbestic points and sedimentation velocity studies demonstrated that poly(c^7I) forms only 1:1 stoichiometric complexes with both poly(C) and poly(br^5C). Poly(C)·poly(c^7I) had a T_m of 49° (0.2 M NaCl, ph7) and poly(br^5C)·poly(c^7I) had a T_m of 86° (0.2 M NaCl, pH7). For comparison purposes, the previously reported poly(br^5C)·poly(I) complex was also prepared.

These complexes were evaluated for antiviral activity and interferon producing ability. Using primary rabbit kidney cells (challenged when appropriate with vesicular stomatitis virus), the following relationships (in order of decreasing activity) obtained when direct inhibition of virus cytopathogenic effect and interferon production in normal, interferon-primed and superinduced (cycloheximide and actinomycin D) primary rabbit kidney cells were measured:

poly(c^7I)·poly(br^5)>poly(I)·poly(br^5C)>poly(I)·poly(C)>poly(c^7I)·poly(C).

On the other hand, if the components of the complexes were administered sequentially followed by measurement of inhibition of virus cytopathogenic effect, then (in order of decreasing activity): poly(I)·poly(C)>poly(c^7I)· poly(br^5C)>

poly(I)·poly(br^5)>poly(c^7I)·poly(C) if either poly(I) or poly(c^7I) were added to the cells first. If either poly(br^5C) or poly(C) were administered first then (in order of decreasing activity):

poly(I)·poly(C)>poly(I)·poly(br^5C)>poly(c^7I)·poly(br^5C)>poly(c^7I)·poly(C)

The results clearly demonstrate that poly(c^7I)·poly(br^5C) and poly(I)·poly(br^5C) are superior to poly(I)·poly(C) as interferon inducers in primary rabbit kidney cells. (P. F. Torrence, E. DeClercq, J. A. Waters, B. Witkop).

Inhibition of RNA Dependent DNA Polymerase by 2'-Modified Polynucleotides.

Single-stranded homopolynucleotides (*e.g.*, poly(U)) have been reported to inhibit the *in vitro* RNA dependent DNA polymerase (RIDP) activity in oncogenic RNA viruses, possibly because of competition between the synthetic homopolynucleotide and the endogenous RNA template for the same enzyme binding site. Although poly(U) appeared to be an effective RIDP inhibitor, it did not prove effective in inhibiting leukemia virus replication in cell culture. The lack of activity of poly(U) in cultured cells may be related to a rapid degradation of the polymer by cellular nucleases. In this view, it became of interest to determine the *in vivo* and *in vitro* activity of polynucleotides which had been rendered resistant to nucleases by substituents at the 2' position. Our studies have revealed that substitutions of 2'-fluoro, 2'-chloro or 2'-azido for the 2'-OH in either poly(C) or poly(U) results in a marked increase of the inhibitory activity of the respective homopolymers (using Maloney murine leukemia virus RIDP assay). Studies are in progress to determine if poly(2'-azido-2'-deoxyuridylic acid) can inhibit the replication of oncogenic RNA viruses in cell culture (P. Torrence, E. De Clercq, B. Janik, F. Eckstein, B. Witkop).

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Synthesis, Chemistry, and Biological Activity of 5-Thiocyanatopyrimidine Nucleosides as Potential Masked Thiols.

The reaction of chlorothiocyanogen (ClSCN) with various derivatives of uracil was investigated as a potential route to 5-mercaptouracils. Reaction of 1,3-dimethyluracil, 1-(β-D-ribofuranosyl)uracil, 1-(2'-deoxy-β-D-ribofuranosyl)-uracil, 1-(β-D-arabinofuranosyl)uracil, 1-(2',3',5'-tri-*o*-chloroacetyl-β-D-ribofuranosyl)uracil, and 1-(2',3',5'-tri-*o*-acetyl-β-D-ribofuranosyl)uracil with ClSCN in acetic acid gave the corresponding 5-thiocyanato derivatives in fair to excellent yields. These 5-thiocyanatopyrimidine nucleosides can be reduced by dithiothreitol, sodium dithionite-2-mercaptoethanol, or glutathione to the biologically active 5-mercaptopyrimidine nucleosides. The intermediate 5-thiocyanatopyrimidine nucleosides themselves show significant biological activity, probably as the result of *in vivo* reduction (T. Nagamachi, J. L. Fourrey, P. F. Torrence, J. A. Waters and B. Witkop).

SECTION ON MICROANALYTICAL SERVICES AND INSTRUMENTATION

Service Function and Instrumentation

Approximately 30 elements and five functional groups are determined routinely by the microanalytical laboratory. The total analyses performed were 11,152. Routine instrumental service continues in the areas of Ir, NMR and mass spectrometry as well as collaborative studies in specialized problem areas. The mass spectrometry service has been upgraded by the acquisition of a Finnigan GC/MS with computer capability. (H. Yeh, N. Whittaker, W. Landis, B. Miller, P. Parisius, A. Wong, B. Baer, and D. F. Johnson).

The Study of Steroid Transformations by Tetrahymena Pyriformis

Previous studies have demonstrated that a variety of steroids of mammalian origin as well as steroid derivatives such as dialkylaminoethoxy steroids inhibit the growth of the protozoa, T. pyriformis, and that this growth inhibition is reversed by the addition of cholesterol. The mode of action of these growth-inhibitory steroids is not definitely known, but inhibition of NADH oxidation and RNA synthesis, and interference with respiration and formation of lipids have been demonstrated. T. pyriformis grown without added steroids produces a pentacyclic triterpnoid alcohol, tetrahymenol as its major unsaponifiable product by a direct, non-oxidative, proton initiated cyclization of squalene. When steroids such as dehydroepiandrosterone are added to cultures, an accumulation of squalene occurs with a corresponding decrease in growth rate of the organism. Since the focus of attention has been on the action of steroids on tetrahymena, the present studies are designed to study the effect of the protozoa on the steroid molecule. All of the substrates incubated (androstenedione, progesterone, 17α-OH-progesterone, testosterone, corticosterone, and pregnenolone) have yielded metabolites, as indicated by column and thin-layer chromatography. Tentative identifications indicate hydroxylation, reduction of a carbonyl group to hydroxyl, and isomerization as possible transformations. Verification of these metabolites is continuing through use of chemical transformations, gas chromatography and mass spectrometry. (N. Lamontagne and D. F. Johnson).

Metabolism of ^{14}C-Labelled Steroids by Adrenals from Rats with a Mammotropic Pituitary Tumor

Earlier studies from this laboratory demonstrated abnormalities in the biogenesis of adrenal steroids by adrenal glands from rats with a mammotropic pituitary tumor (MtT). Reduced conversion of progesterone and qualitative differences of metabolic products was found. Most recent results show that the conversion of pregnenolone to progesterone is also markedly reduced and isolation of metabolites indicates a by-pass biosynthetic pathway than that of normal rat adrenals.

Qualitative and quantitative studies of the metabolites is being carried out with the aid of GC/MS techniques. (B. Weissman and D. F. Johnson)

^{31}P-^{1}H Nuclear Magnetic Resonance Studies of Pyridoxal Phosphates and Derivatives

A modified Varian HA-100 NMR spectrometer, which enables studies of ^{31}P and ^{1}H chemical shifts by double resonance techniques has been used to study the interaction of phosphates of the Vitamin B_6 group and other phosphorous-containing substrates with protonated amines, such as biogenic amines and amino acids. Using this technique and pD titration, it is possible to evaluate these interactions quantitatively. Study of the interaction between the phosphate group of 4-deoxypyridoxine-5'-phosphate (4-dPNP) and different protonated amines shows that basic amino acids and biogenic amines have significant interactions with 4-dPNP. No interactions are observed for acidic or neutral α, β, and γ-amino acids. Among the 19 biogenic amines studied, norepinephrine shows the strongest interaction with 4-dPNP and mono-methyl phosphate. (R. Katz, H. Yeh, and D. F. Johnson).

Nuclear Magnetic Resonance Studies

In addition to some 500 specilized NMR spectra on the HA-100 as a service function, a number of collaborating studies have been carried out (H. Yeh) in this laboratory. The following list summarizes some of the studies completed or in progress:

1. Stereochemical assignments of compounds such as:
   a) the reaction products resulting from action of nucleophiles upon arene oxides (A. Jeffrey and D. M. Jerina).
   b) 2,9β-dimethyl-6,7-benzomorphan (T. Oh-ishi, A. E. Jacobson, R. S. Wilson and E. L. May).
   c) γ-L-glutamyl-S-(trans-1-propenyl)-L-cysteine sulfoxide, isolated from sandal leaves. (T. F. Spande and B. Witkop).
2. ^{1}H and ^{19}F nmr studies of ring fluorinated-imidazoles and histidines (K. Kirk, L. Cohen and J. S. Cohen).
3. Nmr study of mono- and di- substituted benzene oxide-oxepin valence tautomerism. (H. Yagi and D. M. Jerina).
4. Nmr mechanistic study of sny- anti interconversions of aldimines. (H. Ziffer and D. M. Jerina).
5. ^{31}P and ^{1}H nmr studies of interaction between protonated amines, e.g., biogenic amines, amino acids, and organic phosphate containing substrates. (R. Katz and D. F. Johnson).

The Development of Chemical Methods and the Synthesis and Study of Chemical Compounds of Use in the Investigation of Biological and Medical Problems.

Studies are being continued of the photolysis products of N-chloroacetyl-3-(3'-aminopropyl)indole and N-chloroacetyl-3-(4'-aminobutyl)indole. Analytically pure samples have been obtained and spectral studies are being carried out to permit structural assignments. N-chloroacetyl derivatives of compounds such as aniline, benzylamine, β-phenylethylamine, and other related compounds have been prepared and phototitrations carried out. The kinetic data obtained in this way provide useful information on the course and

mechanism of such reactions.

In 1957 the discovery of the fact that the dietary agent, Factor 3, which prevented dietary liver necrosis in the rat, supplied trace amounts of selenium was reported [K. Schwarz and C. M. Foltz, J. Am. Chem. Soc., 79, 3292 (1957)]; at that time it was inferred that selenium might act in intermediary metabolism by participating in oxidation-reduction reactions. This report stimulated a large and growing body of work on the nutritional role of selenium in livestock, laboratory animals, and poultry; on the biological role of selenium and its relationship to that of vitamin E; and on the chemistry of organoselenium compounds. Recently it has been reported that two enzymes involved in oxidation-reduction reactions contain selenium; namely, glutathione peroxidase and glycine reductase, and a selenoprotein which may participate in such reactions has been isolated from lamb muscle [reviewed by T. Stadtman, Science, 183, 915 (1974)]. These facts are some of the considerations which have sustained a continuing interest in the biological roles and the chemistry of selenium compounds. Work on the chemistry of selenium compounds is contemplated. (C. Foltz).

SECTION ON OXIDATION MECHANISMS

The Mechanism of Enzymatic Oxidation and the Role of Reactive Intermediates in Drug Metabolism, Hepatotoxicity and Carcinogenesis

A major portion of the efforts of this section have been directed toward elucidation of fundamental mechanisms of drug metabolism in mammals. To this end, a substantial effort is being directed at the hydrocarbon benzy-[a]pyrene (BP) for two basic reasons: BP is the most prevalent environmental carcinogen to man, and the confusion in the 70 existing papers on the metabolism of this hydrocarbon prove the need for a multidisciplinary approach to the problem. The first phase of this approach has been purely chemical in that 12 phenols, 6 arene oxides, 6 dihydrodiols, and a multitude of quinones were required as reference standards for the enzyme studies. About three fourths of these materials are now in hand. The unusual chemistry of the polycyclic hydrocarbons has prompted development of new synthetic methods for production of these compounds. In particular, halohydrin esters have been exploited as latent precursors to both K-region and non-K-region arene oxides. Specialized methods of phenol synthesis particularly applicable to polycyclic hydrocarbons have been devised. (H. Yagi, P. Dansette, and S. Chu)

In order to explore metabolism of BP, high pressure liquid chromatography has been exploited to separate various classes of metabolites. Preliminary results have been extremely satisfying in that they show prior methods of analysis take into account only 10-30% of the actual metabolites. Studies with liver microsomes, solubilized and purified drug metabolizing systems (P-450 and P-448), and bacteria show marked differences. These differences will be pursued in light of species and organ susceptibility to the action of this carcinogen. A variety of previously undetected metabolites have been isolated. (G. Holder)

A broad screening program for carcinogenic and transforming properties of the suspected carcinogenic metabolites has been undertaken. Testing includes topical application, subcutaneous injection, and transformation of cells in culture. Statistically significant data should be available within 12 months. (D. M. Jerina)

Two classes of enzyme systems, epoxide hydrase and glutathione S-epoxide transferase, are known to detoxify metabolically formed arene oxides which are the suspected bioactivated carcinogens. Phenobarbital was found to induce epoxide hydrase in only four of these strains. 3-Methylcholanthrene induced the hydroxylase in four strains, but did not induce the hydrase. Studies on the mechanism of action of epoxide hydrase have established that these enzymes function by nucleophilic addition rather than acid catalysis as previously thought. Studies with the transferase have established that arene oxides from strained carcinogens are more susceptible to attack and detoxication. Further examination of the chemistry of arene oxides has established marked differences between the kinetics of isomerization and mode of nucleophilic addition of K-region and non-K-region oxides. (J. W. Daly, D. M. Jerina, and P. Dansette)

Interaction of arene oxides with nucleic acid has shown that non-K-region oxides are highly reactive toward DNA in contrast to results of earlier studies. An attempt to correlate reactivity with carcinogenicity is in progress. (D. M. Jerina)

Product ratios in metabolism of chlorobenzene and primary isotope effects in the metabolism of a variety of aromatic hydrocarbons have established that an unsuspected mode of direct hydroxylation occurs in mammalian liver. Hopefully, delineation of the factors involved, will allow an exploitation of this route of drug metabolism which circumvents formation of cytotoxic arene oxides. (H. Selander and J. Tomaszewski)

SECTION ON PHARMACODYNAMICS

Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

More efficient procedures for the isolation of catechol-O-methyltransferase (COMT) have been developed. Antibodies to this enzyme have been prepared and used: to develop a radioimmunoassay for COMT; to study the species and organ distribution of COMT; to study the subcellular distribution and localization of COMT and with immunofluorescent techniques to study its distribution in brain and other tissues. A survey of rat brain with fluorescent anti-COMT revealed the presence of a fine nerve fiber network with clearly outlined dendritic terminals nonuniformly distributed in the areas examined. In addition several CNS structures were highly fluorescent, in particular the cysterna magna. The subcellular distribution and properties of membrane-bound COMT from liver microsomes have been investigated. (C. R. Creveling)

Further studies on the structure-activity correlations on the inhibition of uptake, release, and long term effects on the uptake mechanisms by phenethylamines, phenethanolamines and indoleamines in heart have been

continued. It has been shown that 2-hydroxyphenethanolamines are virtually inactive as inhibitors of the norepinephrine uptake mechanism as compared to the highly effective 2-hydroxyphenethylamines. Consideration of the probable preferred conformation of these compounds derived from molecular orbital calculations suggest that the aromatic 2-hydroxy group is hydrogen bonded to the side chain amino-group. Such a gauche conformer appears to interact strongly with the uptake receptor site except in the presence of a β-hydroxy moiety where the interaction is blocked. The results of this study suggest that both the extended and the gauche conformers of dopamine can interact with the uptake site of noradrenergic nerve terminals in cardiac tissue, but only the extended conformer of norepinephrine has a high affinity. The results rationalize the lack of degenerative effects after administration of 6-hydroxynorepinephrine as contrasted to the marked degenerative effects elicited by 6-hydroxydopamine. (A. Rotman and C. R. Creveling).

The neurodegenerative effect of 5,7-dihydroxy tryptamine has been confirmed by direct morphological examination by electron microscopy of sympathetic nerve in mouse atria and thyroid. The morphological appearance and time course of the degenerative is similar to that induced by 6-hydroxydopamine. The prevention of the long term biochemical and morphological effects of 5,7-dihydroxytryptamine after prior inhibition of monoamine oxidase suggests that the proximate cytotoxic agent is formed by oxidation of the parent compound. 5,6-Dihydroxytryptamine after large dosages causes subepicardial lesions, while ischemic subendocardial lesions are elicited by 6,7-dihydroxytryptamine. 5,7-Dihydroxytryptamine did not elicit such sequelae. (C. R. Creveling)

The binding or radioactive 6-hydroxydopamine, dopamine, 5,7-dihydroxytryptamine, norepinephrine, tryptamine, serotonin and various other amines to bovine serum alubmin has been studied. Only in the case of 6-hydroxydopamine did extensive covalent binding occur. The reaction of 6-hydroxydopamine with albumin result in extensive crosslinking to form a series of albumin polymers ranging from the monomer, dimer, trimer to polymers with molecular weights in excess of a million. This crosslinking reaction occurs thru the sulfhydryl groups of albumin and is absent with albumin where all the available sulfhydryl groups were blocked with N-ethylmaleimide. Radioactive 6-hydroxydopamine is taken up and bound covalently to at least three proteins in mouse and rat atrial preparations *in vitro*. (A. Rotman)

A simple, inexpensive procedure for the labeling of a variety of phenethylamines has been developed consisting of the oxidation of a precursor nitro alcohol to a ketone followed by reduction with sodium borotritide. (A. Rotman)

2,4-Dihydroxy-5-methoxyphenethylamine was synthesized to investigate a recent report that this compound elicits a long lasting depletion of dopamine in the central nervous systems. No affinity for the peripheral norepinephrine-uptake system could be demonstrated. (A. Rotman)

The distribution, metabolism and incorporation into protein of ^{3}H-2-fluorohistidine in the mouse has been examined. 2-Fluorohistidine can substitute for histidine and gives rise to proteins containing 2-fluorohistidine *in vivo*. (C. R. Creveling)

The Role of Cyclic Adenosine Monophosphate in the Central Nervous System

Pretreatment of rats with agents which disrupt noradrenergic input in the central nervous system results in a hypersensitivity of cyclic AMP-generating systems to norepinephrine. Thus, a 1.5-3 fold increase in the accumulation of cyclic AMP elicited by norepinephrine pertains in cerebral cortical slices from animals in which i) noradrenergic terminals have been destroyed by 6-hydroxydopamine ii) central levels of norepinephrine have been reduced by resperine and iii) norepinephrine synthesis has been inhibited by α-methyl-p-tyrosine. Both α- and β-adrenergic receptor-mediated mechanisms appear to be enhanced by such treatments. (R. Dismukes)

Responses of norepinephrine-sensitive cyclic AMP generating systems in combined midbrain-striatal slices of four rat strains correlated positively with spontaneous behavioral activity and negatively with levels of midbrain and striatal tyrosine hydroxylase. Responses of cerebral cortical norepinephrine-sensitive cyclic AMP systems correlated negatively with spontaneous behavioral activity and postively with midbrain and striatal tyrosine hydroxylase. Such correlations were not found with responses of the cyclic AMP-generating systems to isoproterenol, adenosine, veratridine, or of an adenosine and norepinephrine combination. The results suggest that in animals in which the genetically controlled levels of neurotransmitter synthesis and release are low, there has been a compensatory increase in receptor-mediated response systems. The results also provide further evidence for the importance of noradrenergic systems to levels of spontaneous behavioral activity. (P. Skolnick)

Clonidine, a compound proposed to regulate blood pressure, *via* interaction at central presynaptic α-adrenergic receptors was found to be an α-antagonist rather than an α-agonist with respect to generation of cyclic AMP in rat cerebral cortical slices. (P. Skolnick)

A variety of studies have established that depolarization of neuronal structures results in a lowering of levels of ATP in brain slices along with an increase in 5'-AMP and adenosine. The adenosine then appears to stimulate cyclic AMP formation in other morphological entities. Such conclusions are based in part on differential effects of depolarizing agents, metabolic inhibitors and psychotropic agents on levels of ATP, adenosine and cyclic AMP. In addition, studies of brain slices with adenosine deaminase clearly demonstrate that adenosine must be released during depolarization of brain cells into extracellular space before stimulating cyclic AMP formation. Adenosine stimulates intracellular formation of cyclic AMP by interaction with an extracellular receptor as clearly shown by potentiation of the stimulatory effects of low concentration of adenosine by agents which nearly completely block its uptake into the tissue. (J. W. Daly)

A variety of adenine and adenosine analogs have been studied with brain slices as inhibitors of adenine and/or adenosine incorporation and in certain instances as possible precursors of cyclic AMP analogs in the intact cells of these preparations. The fluorescent ethanoadenine and ethanoadenosine analogs were not effectively incorporated into slices. Radioactive N^6-benzyladenosine was readily incorporated into slices, but only the 5'-monophosphate was detected as a product. Radioactive 2'-deoxyadenosine was incorporated into intracellular nucleotides which served as precursors of 3',5'-cyclic phosphates. However, virtually all incorporation of this nucleoside was found to have been preceded by enzymatic hydrolysis to radioactive adenine so that radioactive cyclic AMP rather than radioactive 2-deoxy-cyclic AMP was the major cyclic nucleotide formed on stimulation of prelabeled slices. Radioactive 2-chloroadenine was prepared and is under investigation. (H. Mah)

Serial No. NIAMDD-LC-1
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Oxidation Mechanisms in Metabolic Processes

Previous Serial Number: NIAMDD-LC-1

Prinicipal Investigator: Louis A. Cohen

Other Investigators: John Reepmeyer and Herman Yeh

Cooperating Units: Jack S. Cohen, DCRT-PSL-5.11

Man Years

Total:	0.5
Professional:	0.5
Other:	0

Project Description:

A study of the mechanisms by which energy derived from respiration is ultimately converted into labile bond energy, as in ATP; a study of the mechanisms by which molecular oxygen is activated for direct incorporation into substrate.

Objectives: To demonstrate that when oxygen is bound to metal-chelate systems, it is transformed from the triplet (diradical) state to the singlet (ionic) state and that the direct incorporation of oxygen into substrate involves the electrophilic attack of activated singlet oxygen. To study possible mechanisms for the conservation of oxidative energy by means of chemical models and to apply such knowledge to elucidation of the enzymatic pathways. To devise chemical models for the Mitchell hypothesis--that ATP formation can be coupled to a pH gradient and the free energy available therefrom.

Methods Employed: Infrared and ultraviolet spectroscopy are used to follow rates of reaction of intermediates and to determine structural characteristics. The latter method is also used for quantitative assay of labile materials. Nuclear magnetic resonance spectroscopy is used for structural elucidation of closely related materials. Various chromatographic procedures, particularly on thin layer plates, are used extensively.

Major Findings: Conformationally constrained imidazolecarboxylic acid esters hydrolyze by way of their cyclic acylimidazoles. Acylimidazoles have been implicated in a variety of other reactions of these systems, providing

evidence that high energy bonds can be created enzymatically solely by the trapping of entropic free energy.

Synthesis of the last, but highly elusive, compound in the current series has finally been achieved. As anticipated, this compound shows a high tendency toward utilization of the free energy of water formation as the sole means for generation of high energy bonds. Kinetic studies are in progress to provide a clear model for the Mitchell hypothesis.

Significance to Bio-medical Research and the Program of the Institute: The mechanism by which molecular oxygen is carried into the cell, bound to hemoglobin, has been well elucidated. Its subsequent fate remains obscure. One immediate goal of this project is to determine the mechanisms by which oxygen is activated to a state in which it can attack a variety of relatively inert substrates.

Similarly, the function of ubiquinone in oxidative phosphorylation has yet to be elucidated. It has been shown that oxidative energy is utilized in the conversion of ADP to ATP in mitochondria, ubiquinone being an essential cofactor.

The present findings permit a novel role to be assigned to ubiquinone, Vitamin K and other quinone cofactors.

Controversy regarding the Mitchell hypothesis for mitochondrial synthesis of ATP has continued unabated, since no experiment has been devised to test its validity. The model system under study will be used to attempt to settle the question of whether ATP can be synthesized simply by a rapid change in pH.

Proposed Course: Studies will be continued on bond conservation of oxidative processes and efforts will be made to bridge the gap between the chemical model and the mitochondrion. Studies will be continued on the effects of considerable reduction in the electrostatic gap.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-2
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Chemical Modification and Cleavage of Proteins

Previous Serial Number: NIAMDD-LC-2

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: Robert Jerussi, FDA
Leonard D. Kohn, NIAMDD-LBP

Man Years

Total: 0.3
Professional: 0.3
Other: 0

Project Description:

A study of the chemical modification and fragmentation of polypeptides and proteins by use of selective chemical and physico-chemical reagents. The elucidation of sequence, tertiary structure and protein topology by such methods. Development of techniques for the selective radiolabeling of membrane proteins.

Objectives: To effect the selective modification and splitting of complex peptides by chemical means. To demonstrate the utility of the techniques in splitting long peptide chains into shorter fragments, facilitating sequence determination. To utilize such methods for selective modification of function groups on the surface of a globular protein and thus permit the probing of the surface structure. To modify proteins by selective oxidation or reduction techniques.

Methods Employed: Ultraviolet spectroscopy is used to follow the course and extent of cleavage reactions. Infrared spectroscopy is used to elucidate the structure of reaction products. Paper and column chromatography, ion exchange and high-voltage electrophoresis are used to separate and purify polypeptide fragments. An automatic amino acid analyzer is used to follow the extent of chemical reaction and the purification of peptide fragments. Gas chromatography assists in quantitative end-group assay. Various techniques are used for assay of enzyme activity, including spectral and automatic pH devices. Electrochemical oxidation is effected by use of controlled voltage equipment coupled with automatic pH control.

Major Findings: Continuation of our studies in radioiodination has led to the finding that a two-phase process--substrate in neutral, aqueous buffer and I_2 in chloroform or cyclohexane--may be one of the better ways of effecting radioiodine labelling. The reaction is presumed to occur at the liquid-liquid interface; this method appears not only to enhance the reactivity of I_2 but to permit a level of control in degree and site of iodination heretofor unattainable for many cases.

Significance to Bio-medical Research and the Program of the Institute: Despite the remarkable achievements of the past several years in the X-ray crystallography of proteins, there exists no satisfactory method for determining three-dimensional structure in solution, under conditions of enzymatic activity. The use of a chemically reactive polymer or a platinum electrode, capable of selectively and rapidly modifying functional groups only on the surface, may provide a solution to the problem.

The ability to label membranes in a selective and specific manner with radioactive iodine will serve to facilitate studies on the sites and mechanisms of hormone binding to tissues.

Proposed Course: Information obtained by selective iodination, oxidation and reduction of proteins will be used to provide further information on conformation in solution.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-3
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cleavage of Peptide Bonds by Intramolecular Participation and Mechanisms of Action of Hydrolytic Enzymes

Previous Serial Number: NIAMDD-LC-3

Principal Investigator: Louis A. Cohen

Other Investigators: None

Cooperating Units: None

Man Years

Total: 0
Professional: 0
Other: 0

This project has been temporarily discontinued.

Serial No. NIAMDD-LC-4
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Fluoro Analogs of Enzyme Substrates

Previous Serial Number: NIAMDD-LC-4

Principal Investigator: Louis A. Cohen

Other Investigators: Kenneth L. Kirk, Wakatu Nagai, John Reepmeyer, Y. Takeuchi, Herman Yeh

Cooperating Units: Claude Klee, NIAMDD-LBP
Jack S. Cohen, CR-PSL
Dr. Michael Monahan, Salk Institute, LaJolla, California

Man Years:

Total: 3.9
Professional: 2.7
Other: 1.2

Project Description:

Development of new methods for the synthesis of fluoro analogs of biochemically significant compounds and examination of their properties as enzyme inhibitors and as potential drugs.

Objectives: Fluoro analogs of steroids, purines, pyrimidines and amino acids have been synthesized and tested, several having been found valuable in arthritis and cancer chemotherapy. A larger number have not been prepared because of the limited routes available for the introduction of fluorine. Despite the ubiquitous role of imidazoles in biological systems (RNA, DNA, histidine, histamine, etc.), no fluorinated imidazole has yet been prepared. The initial purpose of this work was to develop a general method suitable for the preparation of fluoroimidazoles, to prepare a series of such compounds, and to test their abilities to serve as enzyme substrates or inhibitors and as replacement for histidine in proteins or for purines in DNA and RNA.

Methods Employed: Ultraviolet irradiation is used as the key step in the introduction of fluorine. Infrared, ultraviolet, mass, and nmr spectroscopy are used to follow the course of reactions and to elucidate product structure. Various chromatographic procedures are used for analysis and purification of materials.

Major Findings: Continued study in fluoroimidazole synthesis has now led to 4,5-difluoroimidazole, an inhibitor of cyclic AMP phosphodiesterase. 2-Fluorohistidine has been found to incorporate into newly synthesized rat liver protein, but to become covalently bound to rat brain histamine receptor sites at the same time. Ribonuclease-S-peptide containing 4-fluorohistidine has been synthesized and found to reaggregate with S-protein. The enzymatic inactivity of the complex proves the general base role of histidine-12 in the catalytic process. 2-Fluorourocanic acid has been found an extremely potent inhibitor of urocanase. This compound will be used to investigate the role of urocanic acid in mammalian skin. 2-Fluorohistidine has proved to be a potent inhibitor of E. coli. 4-Fluoroimidazole-5-carboxamide ribotide has been found an interesting and possibly useful antiviral agent.

Significance to Bio-medical Research and the Program of the Institute: Past experience with fluoro analogs (fluorocitrate, fluorocortisone, and fluorouracil) warrants a search for other medicinally useful fluoroanalogs. Recent findings on the value of histidine in arthritis therapy, of imidazole-carboxamide in skin cancer therapy, and of histamine as a false neurotransmitter emphasize the magnitude of directions in which fluoroimidazoles may be examined clinically.

Proposed Course: Fluoroimidazoles already prepared will be submitted for pharmacological testing and will be examined as enzyme substrate analogs. Additional and more complex compounds will be prepared for the same purpose. The photochemical method of synthesis will be applied to other types of heterocyclic systems, in addition to imidazole. Fluorine nuclear magnetic resonance spectroscopy will be used to study enzyme-substrate complexes with a view to aiding in the elucidation of mechanism of enzymes such as histidine deaminase.

Honors and Awards: None

Publications:

Nagai, W., Kirk, K. L., and Cohen, L. A.: Synthesis of 2-Amino-L-histidine and 2-Aminohistamine. J. Org. Chem., 38, 1971-1974, 1973.

Kirk, K. L. and Cohen, L. A.: Photochemistry of Diazonium Salts. I. Synthesis of 4-Fluoroimidazole, 4-Fluorohistamine, and 4-Fluorohistidine. J. Amer. Chem. Soc., 95, 4619-4624, 1973.

Kirk, K. L. and Cohen, L. A.: Photochemistry of Diazonium Salts. II. Synthesis of 2-Fluoro-L-histidine and 2-Fluorohistamine, and the Halogen Mobility of 2-Fluoroimidazoles. J. Amer. Chem. Soc. 95, 8389-8392, 1973.

Kirk, K. L. and Cohen, L. A.: Photochemistry of Diazonium Salts. III. A New and Facile Synthesis of 4-Fluoroimidazoles. J. Org. Chem., 38: 3647-3648, 1973.

Serial No. NIAMDD-LC-5
1. Chemistry
2. Biochemical Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: General Principles of Enzyme Catalysis and Simulation

Previous Serial Number: NIAMDD-LC-5

Principal Investigator: Louis A. Cohen

Other Investigators: Paul S. Hillery

Cooperating Units: None

Man Years

Total: 1.3
Professional: 1.3
Other: 0

Project Description:

A study of the principles used by enzymes to accelerate chemical reactions. Model compounds are designed and synthesized which are capable of undergoing various reactions at rates comparable to those of enzymes, and under the same reaction conditions. Recognition of the principal devices used by enzymes will permit the design of more effective drugs and enzyme inhibitors or substrates.

Objectives: The rates of enzyme-catalyzed reactions exceed those of simple test-tube analogs by factors of 10^{10}-10^{18}. In order to account for such a phenomenal effect, model compounds which duplicate one or more of an enzyme's special powers are studied to learn more about the vastly complex protein catalyst. In the belief that the principal enzymatic device is stereopopulation control (near perfect orientation of functional groups), model compounds have been designed and synthesized in which rotation of covalent bonds has been severely restricted. By bringing two functional groups into very close proximity and orientation, rates of reaction comparable to those of enzymes can be achieved in the test tube.

Methods Employed: Spectroscopic methods are used to elucidate the structures of synthetic products and to follow rates of reaction. Various chromatographic procedures are employed for analysis and purification.

Major Findings: Studies have continued on the kinetics of cyclization of alkylated homophthalic acids. Three important developments have emerged from the study this past year. (1) A linear correlation can be shown between log k for cyclization and the pK of the general acid catalyst, covering the range

from acetic to perchloric acid, a spread of 12 pK units. This range is at least twice as great as that attainable in any previous study of general acid catalysis. (2) Catalysis by general base has also been demonstrated; such catalysis is possible only if breakdown of tetrahedral intermediate is rate limiting. For the first time, general catalysis has been used to prove conclusively the identity of the rate-determining step in a carbonyl reaction. (3) Removal of one methyl group from the 'trialkyl lock' reduces the rate enhancement factor only by 50%. This observation eliminates once and for all any arguments that the enormous rate enhancements observed in this laboratory are due to relief of steric compression; in the last case mentioned, steric compression cannot exist. It now appears clear that 'stereopopulation control' is indeed achieved by freezing of rotational isomerism and that it does provide the best model for the enzyme-substrate complex available to date.

Honors and Awards: Smith, Kline and French Lecture in Medicinal Chemistry, University of California, Irvine, California, May 23-24, 1974.

Publications:

Borchardt, R. T. and Cohen, L. A.: Stereopopulation Control. IV. Facilitation of Intramolecular Conjugate Addition of the Hydroxyl Group. J. Amer. Chem. Soc., 95: 8308-8313, 1973.

Borchardt, R. T. and Cohen, L. A.: Stereopopulation Control. V. Facilitation of Intramolecular Conjugate Addition of the Aldehyde Group. J. Amer. Chem. Soc., 95: 8313-8319, 1973.

Borchardt, R. T. and Cohen, L. A.: Stereopopulation Control. VI. Conformational Selection of Alternative Oxidation Pathways. J. Amer. Chem. Soc., 95: 8319-8326, 1973.

Serial No. NIAMDD-LC-6
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Synthesis of Carbohydrate Derivatives for Biomedical Research

Previous Serial Number: NIAMDD-LC-6

Principal Investigator: H. G. Fletcher, Jr. (deceased 10/19/73) and R. K. Ness

Other Investigators: M. Pokorny, E. Zissis and E. W. Tracy

Cooperating Units: (until 10/19/73) Dr. N. Pravdić, Department of Organic Chemistry and Biochemistry "Rudjer Bosković" Yugoslavia (under P.L. 480), Harry W. Diehl, former colleague, now retired.

Man Years:

Total: 3
Professional: 2
Other: 1

Part A. 2-Acetamido-2-deoxyaldonic Acids

Project Description:

Objective: To investigate the chemistry and biochemistry of the 2-acetamido-2-deoxyaldonic acids and their derivatives.

Major Findings: Brief treatment of 2-acetamido-2-deoxy-D-gluconic acid with boiling acetic acid affords crystalline 2-acetamido-2-deoxy-D-gluconic-1,4-lactone. This is the first time that this potential inhibitor of "β-N-acetyl-D-glucosaminidase" has been isolated in a pure state. The lactone may also be prepared through hydrolytic cleavage of the isopropylidene group in 2-acetamido-2-deoxy-5,6-O-isopropylidene-D-glucono-1,4-lactone. Treatment of this isopropylidene compound with p-toluenesulfonylchloride in pyridine induces a β-elimination yielding an unsaturated lactone, 2-acetamido-2,3-dideoxy-5,6-O-isopropylidene-D-erythro-hex-2-enono-1,4-lactone. Contrary to previous experience, at least some of the components of the products from the oxidation of 2-acetamido-2-deoxy-D-glucose are susceptible to alcoholysis with methanol and with ethanol. Although complete opening of the lactone ring had been previously observed to involve a long period of time, partial alcoholysis seems to occur immediately. Three methyl 2-acetamido-2-

deoxy-D-gluconates and one ethyl ester were isolated as isopropylidene derivatives from the oxidation of 2-acetamido-2-deoxy-D-glucose after the product was exposed to methanol or ethanol and subsequently isopropylidenated. (N. Pravdić, E. Zissis, M. Pokorny, and H. G. Fletcher, Jr.)

The above mentioned crystalline 2-acetamido-2-deoxy-D-glucono-1,4-lactone and its corresponding acid (recently obtained crystalline for the first time in this laboratory) have been examined for inhibitory activity against β-N-acetyl-D-glucosaminidase from bull epididymis. These have been compared to the known crystalline 2-acetamido-2-deoxy-D-glucono-1,5-lactone. The 5,6-isopropylidene derivative of the 1,4-lactone and the 4,6-isopropylidene derivative of the 1,5-lactone were also tested. The overall results, especially from the isopropylidene derivatives, indicate that the 1,5-lactone is far more active as an inhibitor (M. Pokorny and E. Zissis).

Oxidation of N,N^1-diacetylchitobiose with bromine-water in the presence of cadmium carbonate has yielded an acid, which in turn forms a cadmium salt with cadmium carbonate. The acid can be regenerated with hydrogen sulfide. The lactone, formed by concentration of an aqueous solution of the acid, has not yet been obtained in a crystalline or in an analytically pure state, but the preparation has shown a moderate inhibition of the β-N-acetyl-D-glucosamindase from bull epididymis, while in the Avena coleoptile section test it is also active (R. K. Ness, M. Pokorny, and E. Zissis).

Significance to Biomedical Research and to the Program of the Institute: There has been an increase in the understanding of the chemistry and in some of the biochemical properties of the 2-acetamido-2-deoxyaldonic acids and their derivatives.

Part B. Aldonolactones

Project Description:

Objectives: To investigate the chemistry and biochemistry of the aldonolactones and their derivatives.

Major Findings: A biological assay has been employed for the first time in considering the effects that various aldonolactones might have on plant growth. It has now been shown that, in the standard Avena coleoptile section test, of the 38 lactones of aldonic acids and derivatives thereof, all but D-ribono-1,4-lactone and L-galactono-1,4-lactone showed some inhibitory activity. The ratio of inhibition varied however as to the ring size of the lactone as well as to the type and configuration of the sugar from which the lactone had been synthesized. The results showed that, in the same sugar series, the 1,5-lactones were better inhibitors than the corresponding 1,4-lactones. Part of the activity of 1,4-lactones might be attributed to their corresponding 1,5-lactones that spontaneously arise in the process of equilibrium in solution. Only in the case of D-glucaro-1,4-lactone was the inhibition similar to its 1,5-lactone. This effect may be due to a rapid equilibrium of the two lactones in solution.

Although certain herbicides possessing a lactone structure are known to inhibit indoleacetic acid activity, in the coleoptile no significant difference was observed in the effect of aldonolactones on the growth inhibition of coleoptiles in the presence of 2% sucrose or various concentrations of indoleacetic acid. Plant sections, treated with an aqueous solution of a lactone and the lactone removed by washing, were not permanently inhibited in their growth by the lactone; therefore, these lactones may be considered to be reversible growth inhibitors, a trait observed in nonsugar herbicides possessing a lactone group.

The results indicate that retarded coleoptile growth in the presence of aldonolactones is the consequence of a blocked glycosidase system in the tissues (M. Pokorny).

Utilizing the procedure recently developed in this laboratory for the preparation of aldonic acids and their lactones (oxidation of the aldose with bromine-water in the presence of cadmium carbonate), cellobiono-1,5-lactone was prepared in crystalline form for the first time. It was found, as a growth inhibitor in the Avena coleoptile section test to be as active as any of the most active aldonolactones thus far observed (H. Diehl, M. Pokorny, E. Zissis, R. K. Ness, and H. G. Fletcher).

Significance to Biomedical Research and to the Program of the Institute: Sugar lactones are known to inhibit specific glycosidases. Cellobiono-1,5-lactone has been shown to be a very active inhibitor of plant growth. Aldonolactones and aldonic acids have been found to have an inhibitory effect upon certain glycosidases. Apart from the general interest in these enzymes, there is a specific interest centered around the sphingo lipidoses (i.e., Fabry's disease, etc.). Even the rudimentary chemistry of these inhibitors is ill understood and this study will hopefully clarify that situation to a large extent.

Part C. Chemistry of Ketoses

Project Description:

Objectives: To devise methods of synthesizing the cis form (C2-C3) of a D-fructofuranyl nucleoside--initially 9-β-D-fructo-furanosyl adenine.

Present Status: 3-O-Benzyl-D-fructose is being prepared as starting material (E. W. Tracy and R. K. Ness).

Although the keto-D-fructose pentabenzoate has been known since 1933, no pentabenzoate of a ring form of D-fructose has previously been recognized. A pyranose and a furanose form have now been prepared under forcing conditions during the benzoylation (H. W. Diehl and R. K. Ness).

Significance to Biomedical Research and to the Program of the Institute: Psicofuranine is a ketohexosyladenine with the β-ribo configuration in the sugar ring. It is cytotoxic and toxic. The only known 2-keto hexosyl adenine

with the arabino ring configuration is an α isomer. The synthesis of the β isomer (i.e., the β fructofuranosyl adenine) as a potential anti-cancer drug, is therefore of interest.

Publications:

Pokorny, M.: Growth Inhibitory Activity of Aldonolactones in Avena Coleoptile Section Test. Acta Bot. Croat., in press.

Pravdić, N., Zissis, E., Pokorny, M., and Fletcher, Jr., H. G.: Synthesis of 2-acetamido-2-deoxy-D-glucono-1,4-lactone and some isopropylidene acetals of 2-acetamido-2-doexy-D-gluconic acid derivatives.: Carbohyd. Res., 32 (1974).

Diehl, H. W., Pokorny, M., Zissis, E., Ness, R. K., and Fletcher, Jr., H. G.: Crystalline cellobiono-1,5-lactone: Its preparation and growth inhibitory activity in the Avena Coleoptile Section Test, in preparation.

Diehl, H. W. and Ness, R. K.: D-Fructopyranose and D-Fructofuranose Pentabenzoates, in preparation.

Pokorny, M. and Zissis, E.: A study of the inhibitory activities of 2-acetamido-2-deoxy-D-gluconolactones and related compounds against β-N-acetylglucosaminidase, in preparation.

Serial No. NIAMDD-LC-7
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunochemistry and Reactions of Carbohydrates

Previous Serial Number: NIAMDD-LC-7

Principal Investigator: C. P. J. Glaudemans

Other Investigators: M. E. Jolley, M. Pokorny, E. Zissis, R. Ness

Cooperating Units: M. Potter, S. Rudikoff (NCI)

Man Years

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: We are interested in studying the interaction of carbohydrate antigens with homogeneous antibodies. We are presently studying the combining region of two homogeneous IgA's with anti-β-D-galactan specificity. We have greatly increased our knowledge on the nature of the combining region of these two immunoglobulins and work now in progress will further elucidate the nature of binding between haptens and immunoglobulins.

Methods Employed: The usual techniques of chemistry and biochemistry are employed, such as affinity chromatography employing hapten-coupled sepharose columns, fluorescence titration, organic synthesis, gas-liquid chromatography, mass spectroscopy, etc.

Major Findings:

1. Binding Studies: The active sites of two of our eight anti-β-(1→6)-D-galactan myeloma proteins were studied by determination of their binding constants (K_a values) with a variety of derivatives of D-galactose. The binding constants between the various haptens and the two proteins (X-24 and J-539) were determined by the method of fluorescence enhancement, a method developed by us (and independantly and simultaneously by R. Pollet and H. Edelhoch), which is vastly superior to equilibrium dialysis, the foremost alternative. Fluorescence titration is quick, simple, requires no derivatized haptens and is capable of great accuracy and reproduceability even at K_a values of 10^2. In addition, very small quantities of protein are required.

By the determination of the K_a values of a variety of derivatives of D-galactose with these proteins certain deductions could be made with regard to the requirements in the hapten for binding (by comparing their free energies of binding).

a. The terminal non-reducing residue must be in the galacto-conformation, but the C_5 hydroxymethyl group is not essential for binding.

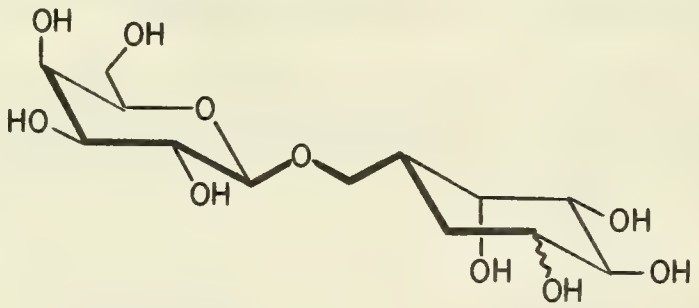
b. The glycosidic linkage must not be α. Haptens with a β-linked aglycone moiety, or with no aglycone moiety bind, but an α-linked aglycone moiety results in non-binding.

c. In oligomers of β(1→6)-linked D-galactose the terminal non-reducing residue binds the strongest, then the second residue, then the third residue. The terminal reducing reside in galactotetraose (residue number four) contributes little to the overall binding of this oligosaccharide.

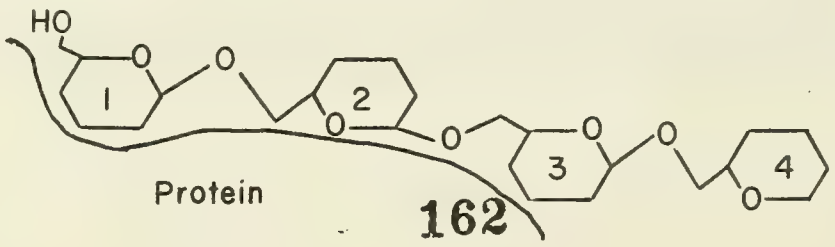
d. The portion of the disaccharide (β-D(1,6) galactodiose) molecule from the glycosidic oxygen atom to C_1 of the second (reducing) residue contributes significantly to the binding of that sugar.

e. C_2, C_3 and C_4 and their associated hydroxyl groups of the reducing residue (residue two) contribute littler to the binding of the disaccharide.

Subtle differences were noted in the active sites of the two proteins, but on the whole they were very similar. From these studies the main areas of binding on the disaccharide molecule could be mapped out:



As can be seen the main areas of binding are located down one side of the molecule. This indicates that the binding sites on the proteins are in the form of surfaces rather than clefts. This is consistent with the binding of the tetrasaccharide:

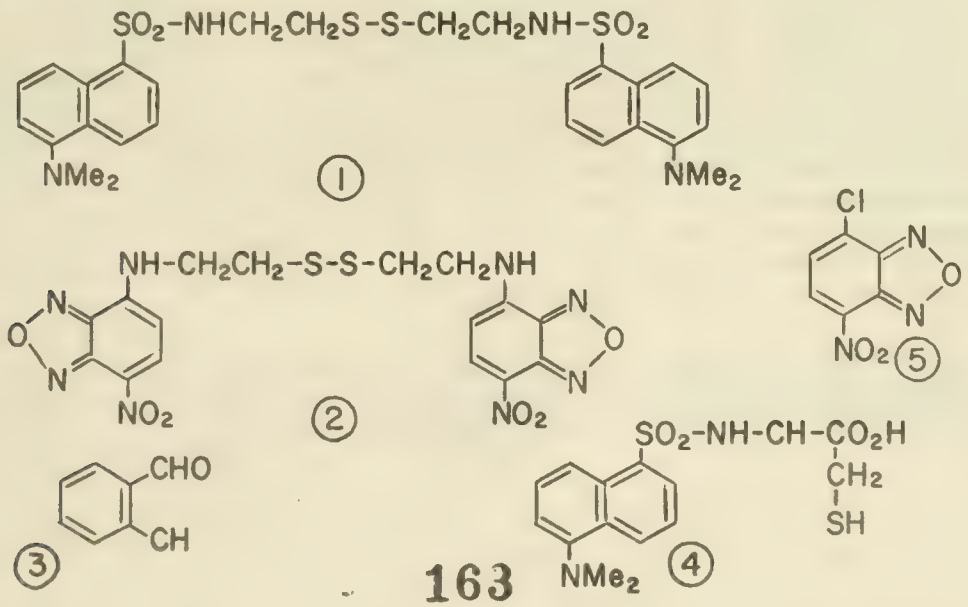


As can be seen, in the postulated conformation (determined by model building) residue four (the terminal reducing residue) juts sharply away from the protein and so cannot contribute much to the binding of the tetrasaccharide, in agreement with the experimental data. To test our hypothesis that only the front of the disaccharide ligand β-D-(1,6) galactodiose binds to these homogeneous antibodies we synthesized methyl 6-O-acetyl β-D-galactopyranoside (Zissis). If correct this ligand should bind with approximately the same strength as the methyl β-D-galactopyranoside (no bulky acetate in the "rear" of the molecule where binding presumably does not take place.) Fluorescence titrations have shown this to be so (Zissis).

Further work is continuing in this field. Galactose-β-(1→6)-glucose and galactose-β-(1→6)-mannose are currently being prepared to support our contention that C_2, C_3, and C_4 and their associated hydroxyl groups of residue 2 contribute little to the binding of the disaccharide. Other D-galactose derivatives are also being prepared, modified at C_6 and C_1 in order to refine our model.

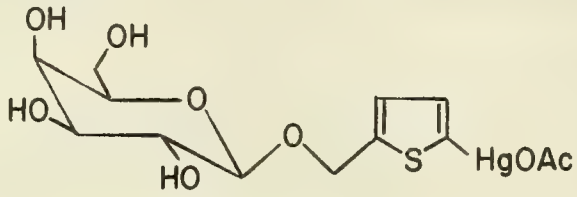
Attempts were made to affinity-label X-24 and J-539 proteins with the compounds 2',3'-epoxypropyl-β-D-galactopyranoside and N-bromoacetyl-p-aminophenyl-thio-β-D-galactopyranosides. These attempts have so far been unsuccessful. Photoaffinity labels are being prepared which might prove more successful.

2. Conformational Changes on Hapten Binding: It was discovered that proteins J-539 and X-24 possess one free sulphydryl group by reaction with ^{14}C-labeled N-ethyl-maleimide. This sulphydryl group is probably on the Fc portion of the molecule since all the cysteine residues in the Fab fragment appear to be tied up in disulphide linkages. Attempts were made to attach a fluorescent label onto this sulphydryl residue by means of a variety of reagents and so detect a slight conformational change in the Fc fragment on binding hapten.



All but o-phthalaldehyde (3) proved only partially successful, probably due to steric and solubility factors. However, the reaction with o-phthalaldehyde is not understood and it may not be reacting with a sulphydryl group. Further investigation into this reaction is proceeding. Studies are proceeding into the location of the free sulphydryl group.

3. X-ray Crystallographic Studies on the Anti-galactan: J-539 Fab' fragment has crystallized and x-ray studies are being performed by Dr. Davies' group. The mercurated hapten has been prepared by us and



⑨

causes significant quenching (∿50%) of the tryptophanyl fluorescence of proteins X-24 and J-539. The binding constant of this compound (9) (as determined by fluorescence quenching titrations) appears to be similar to that of the disaccharide galactose-β(1→6)-galactose. X-ray studies are presently in progress on the complex between this compound and J-539 Fab' in the crystalline state.

4. Specificity of Antigalactan IgA's: Further studies into the specificity of these proteins have been made. Larch arabinogalactan, a polysaccharide possessing a backbone of β(1→3) linked galactopyranoside units with short side chains of β(1→6)-linked galactopyranoside oligomers and single side chains of L-arabinose precipitates with these proteins. Mammalian lung galactan also precipitates, indicating the presence of β(1→6)-linked galactopyranoside side-chain residues in the latter polysaccharide. Arabinogalactan, purified by passage down a DEAE-cellulose column, was periodate oxidized, reduced ($NaBH_4$) and hydrolysed with mild acid. A material was obtained which is presumably a linear β(1→3) galactan, since all the side-chain residues would be destroyed by this treatment. This material does not precipitate with these proteins indicating that the specificity is for the β(1→6)-linked galactopyranoside side chains. However, the β(1→3) galactan does cause an increase in the tryptophanyl fluorescence of proteins X-24 and J-539, indicating binding, but the affinity of these proteins for this material appears to be very low.

Work is presently being conducted into the synthesis of two spin-labeled galactosides with a view to shedding further light on the binding of haptens to these proteins by E.S.R. measurements.

Professor C. Schuerch at Syracuse, an expert on the synthesis of rigorously defined stereoregular polysaccharides is presently applying for funds to start a collaborative program investigating the specificity of these immunoglobulins with our group.

5. N-acylhexosaminyl-rhamnose Ligands: Streptococcal polysaccharides can induce an immune response that yields antibodies of restricted heterogeneity. These antibodies could possibly be purified to homogeneity, and we are interested in studying the binding of antibodies thus obtained with streptococcal antigenic determinants such as N-acetylglucosaminyl (and galactosaminyl)-rhamnose.

A crystalline tri-O-benzoyl-d-rhamnopyranose, prepared in 1951 in this laboratory, but never completely characterized, is being investigated as a potential starting material. The compound has been mesylated and the product converted to a methyl dibenzoyl-monomesyl-D-rhamnoside. It will be debenzoylated and oxidized with sodium periodate to ascertain the position of the hydroxyl group in the original crystalline rhamnopyranose tribenzoate. From the mother liquor of the crystalline D-rhamnopyranose tribenzoate now being characterized was obtained another benzoylated rhamnose compound (amorphous). This, too, is being checked out as a starting material for the preparation of one of the desired disaccharides (E. Zissis and R. K. Ness).

Significance to Biomedical Research and the Program of the Institute: The Section on Carbohydrates, with its knowledge of immunochemistry and structural carbohydrate chemistry is in a position to make a real contribution to the study of the genetics and the diversity of the immune response, employing this unique set of immunoglobulins. The specificity of antibodies and the problem of "the economy of the immune response" can be studied very well using this set of immunoglobulins.

Proposed Course of Project: We have made considerable progress in the past year towards the objections mentioned in last year's report. The combining region of antibodies is better understood largely due to the work in our group. In the future we will try to do extensive studies on affinity labeling of homogeneous antibodies, and on amino acid sequencing in order to come to knowledge of the fine structure (together with Dr. Davies' section which will be studying the x-ray diffraction) of one of these IgA's.

Honors and Awards: None

Publications:

Jolley, M. E., Rudikoff, S., Potter, M. and Glaudemans, C. P. J.: Spectral Changes on Binding Oligosaccharides to Murine IgA Myeloma Proteins. Biochemistry 12, 3039 (1973).

Rudikoff, S., Mushinski, E. B., Potter, M., Glaudemans, C. P. J. and Jolley, M. E.: Six Balbe/c IgA Myeloma Proteins That Bind β(1→6) D-galactan. Partial amino acid sequences and idiotypes. J. Exp. Med. 138, 1095 (1973).

Glaudemans, C. P. J., Jolley, M. E. and Potter, M.: Mammalian Lung Galactan: Carbohyd. Res. 30, 409 (1973).

Jolley, M. E. and Glaudemans, C. P. J.: An Accurate Method to Determined the Association Constant Between Carbohydrate Ligands and Proteins. Carbohyd. Res., in press.

Jolley, M. E., Glaudemans, C. P. J., Rudikoff, S. and Potter, M.: Structural Requirements for the Binding of Derivatives of D-galactose to Two Murine IgA Immunoglobulins. Biochemistry, submitted for publication.

Glaudemans, C. P. J.: The Binding of Polysaccharide Antigens to Homogeneous Murine Immunoglobulins. Advances in Carbohyd. Chem. and Biochem., submitted for publication.

Glaudemans, C. P. J.: Hewitt Grenville Fletcher, 1917-1973. Carbohyd. Res. 31, 157 (1973).

Glaudemans, C. P. J.: Hewitt Grenville Fletcher. Advances in Carbohyd. Chem. and Biochem., submitted for publication.

Serial No. NIAMDD-LC-8
1. Chemistry
2. Carbohydrates
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Preparation of 1-O-acyl glucuronic acids (of Unsaturated Acids)

Previous Serial Number: None

Principal Investigator: C. P. J. Glaudemans

Other Investigators: N. Roy, Council of Scientific and Industrial Research, India

Man Years:

Total: .5
Professional: .5
Other: 0

Project Description:

Objective: To develop a general method for the preparation of 1-O-acyl glycoses having unsaturated acids esterified to C-1 of the carbohydrate moiety, and ultimately, to synthesize bilirubin diglucuronide.

Major Findings: When benzyl 6-O-trityl-β D-glucopyranoside is treated with chloroacetic anhydride and pyridine it becomes fully acylated. The resulting benzyl-6-O-trityl-2,3,4 tri-O-chloroacetyl-β-D-glucopyranoside can be detritylated with 1 mole of hydrobromic acid, to yield benzyl 2,3,4 tri-O-chloroacetyl-β-D-glucopyranoside. This compound shows very facile chloroacetyl migration from O-4 to O-6. However, if the detritylated material is immediately oxidized with permanganate it yields benzyl 2,3,4 tri-O-chloroacetyl β-D-glucuronide. Hydrogenation of this (H_2/Pd) gives the reducing glucuronic acid (blocked at position 2,3,4 with chloroacetyl groups) as a crystalline substance. This compound can be used to prepare 1-O-acyl derivatives from which the chloroacetate can be removed by thiourea in methanol. If unsaturated acids are used to form 1-O-acyl derivatives, the use of the chloroacetyl blocking group allows removal of these groups with preservation of the unsaturation in the 1-O-acyl compound.

Significance to Biomedical Research and the Program of the Institute: The synthesis of bilirubin diglucuronide has never been accomplished. This is due to the fact bilirubin has unsaturated (olefinic) bonds. Chemical coupling with glucuronic acid via this acid blocked at position 2,3 and 4 by benzyl groups is not feasible, as removal of these benzyl groups (H_2/Pd) would also cause reduction in the bilirubin moiety. Blocking at C2, 3 and 4 of the

glucuronic acid moiety with acetyl groups, prior to coupling also is impossible, as removal of these esters would sever the ester linkage with the bilirubin moiety. Some years ago our group (Bertolini & Glaudemans) developed the use of the chloroacetyl group for blocking purposes in carbohydrate chemistry, and these chloroacetates can be removed with thiourea under neutral conditions. This has now led to a feasible intermediate for the preparation of bilirubin diglucuronide.

Proposed Course: It is suggested to study the condensation of 2,3,4 tri-O-chloroacetyl D-glucuronic acid with a variety of unsaturated esters and then with bilirubin.

Honors and Awards: none

Publications:

N. Roy and C. P. J. Glaudemans: The synthesis of 2,3,4-tri-O-chloroacetyl-D-glucuronic acid, and of its methyl ester. Manuscript in preparation.

Serial No. NIAMDD-LC-9
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Testing for Analgesic Activity, Dependence Liability and Narcotic Antagonism

Previous Serial No.: NIAMDD-LC, #8

Principal Investigator: Everette L. May

Other Investigators: E. L. Atwell

Cooperating Units: Animal Production Section, NIH; Department of Pharmacology University of Michigan and Medical College of Virginia

Man Years:
Total: 1.5
Professional: 0.5
Other: 1.0

Project Description:

Objectives: 1) To determine analgesic activity, acute toxicity, narcotic antagonist activity and abuse potential of compounds that act on the CNS. 2) To render advice to the DEA, FDA, WHO, SAODAP and the pharmaceutical industry.

Methods Employed: Hot-plate and Nilsen methods of assay for analgesia and 24-hour acute toxicity determinations on white, male, Caeserian-derived, GP mice. Abuse potential and properties of narcotic antagonism were determined in Rhesus monkeys and, if warranted, in man at the Addiction Research Center, Lexington.

Major Findings: Eighty-nine compounds were tested in the hot-plate (total number of experiments 323), 27 by the Nilsen method (102 experiments). The acute (24-hr) toxicity of six compounds (20 experiments) was determined. In this way compounds to be further studied and starting doses were determined. Forty-nine reports on physical dependence capacity (single-dose) experiments were received from Michigan on the more interesting compounds. Five older drugs were subjected to primary-addiction studies and complete reports on these were made available. Two are of particular interest. 1) The N-amyl

homolog (synthesized by Medicinal Chemistry, L.C.) of the heroin-like ketobemidone, three times as potent analgesically as the parent, produced no physical dependence in Rhesus monkeys, in fact exhibits atypical properties of narcotic antagonism.

2) N-cyclopropylmethyl-2-hydroxymorphinan, nearly as powerful as morphine, also will not cause physical dependence in monkeys. This is the first 2-hydroxymorphinan to show appreciable analgesic activity.

Significance to Biomedical Research and the Program of the Institute: Constitutes a systematic and concerted effort toward providing safer pain-relieving agents and their antagonists and toward solution of the problem of drug addiction.

Proposed Course: To continue essentially as before with expansion to broader-based, greater in-depth studies by virtue of a collaborative arrangement just begun with NIDA, ADAMHA.

Publications: None

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Titles: 1) Molecular Orbital Calculations on the Conformations of Biogenic Amines.
2) Hansch Analysis of 6,7-benzomorphan agonists.
3) Syntheses of narcotic agonists and antagonists.
4) Study of the in vitro "receptor" binding of narcotics and their antagonists.

Previous Serial No.: Same

Principal Investigator: Arthur E. Jacobson

Other Investigators: W. Klee, R. Katz, S. R. Heller, H. J.C. Yeh, E. L. May, C. R. Creveling, A. Rotman, R. S. Wilson, T. Oh-ishi, S. Shiotani

Cooperating Units: NIMH (W. Klee), Andrulis Research Corp. (R. Katz), E.P.A. (S. R. Heller)

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To calculate, by the Complete Neglect of Differential Overlap (CNDO) method, the minimum energy conformations of compounds which experimentally show different affinities for the site at which norepinephrine is taken up in noradrenergic nerve terminals.

2) To determine by a regression analysis technique, whether experimentally determined analgesic activity can be correlated with calculated physico-chemical parameters for a series of 6,7-benzomorphans.

3) A. To synthesize various derivatives of morphine in order to develop substitutes for heroin which would be longer-acting, as part of an overall program in which heroin has been suggested as one treatment moiety for certain types of heroin-addicts.

B. To synthesize new narcotic antagonists.

4) To determine the mode of action of narcotic antagonists and agonists by using an in vitro mixture containing the opiate "receptors"

and through the use of cell-culture preparations shown to have "receptors" for these opiates.

Methods Employed: The usual organic, biochemical, instrumental and computer techniques.

Major Findings: 1) CNDO calculations have shown that there are differences between the minimum energy conformations of those compounds which do, and those which do not have affinity for the site at which norepinephrine is taken up in noradrenergic nerve terminals.

2) An equation has been developed from the Hansch analysis of 60 known 6,7-benzomorphan analgesics from which a reasonably accurate calculation of analgesic activity can be made, using standard physico-chemical parameters, for new, theoretical, analgesics of this type.

3) A. A series of O-substituted morphine derivatives have been prepared; these have been described in detail in the Project Report by E. L. May.

B. An N-isopropyl-2'-hydroxy-5,9α-dimethyl-6,7-benzomorphan has been prepared and is undergoing in vitro and in vivo evaluation as, possibly, a new type of narcotic antagonist.

4) Fluorescent derivatives of some 6,7-benzomorphans have been prepared and have been found to bind only weakly to narcotic "receptors" in in vitro experiments.

Significance to Biomedical Research and the Program of the Institute:

1) Some insight has been gained about the uptake mechanism for biogenic amines at noradrenergic nerve terminals.

2) The development of molecular structure-(as related to various physico-chemical parameters) biological activity relationships among one of the most important series of analgesics could provide further insight into in vivo interactions of relatively small molecules with macromolecular systems. It further provides a useful mathematical method of calculating the in vivo analgesic activity of molecules which have not, as yet, been synthesized.

3) Examination of the in vitro interactions between analgesic, or narcotic antagonist, compounds and their "receptors" (from whole brain preparations or on cultured cells) could lead to a more fundamental understanding of the biological activity of these molecules.

Proposed Course: A continuation and extension of the subjects in the Project Titles.

Honors and Awards:

Appointment to membership on the Committee on Problems of Drug Dependence of the National Research Council, National Academy of Sciences.

Invited Lecture at the VII Jerusalem Symposium on Molecular and Quantum Pharmacology.

Publications: May, E. L. and Jacobson, A. E.: First International Conference on Narcotic Antagonists, in press, 1974.

Katz, R. and Jacobson, A. E.: A molecular orbital study of 2,4,5-trihydroxyphenethylamine and related polyhydroxyphenethylamines. Mol. Pharmacol. 9: 495-504, 1973.

Katz, R., Heller, S. R., and Jacobson, A. E.: A molecular orbital study of norepinephrine and 3,4-dihydroxyphenethylamine: A re-evaluation of structure-activity relationships in norepinephrine. Mol. Pharmacol. 9: 486-494, 1973.

Oh-ishi, T., Jacobson, A. E., Wilson, R. S., Yeh, H. J.C., and May, E. L.: Synthesis of 2,9β-dimethyl-6,7-benzomorphan, J. Org. Chem., in press, 1974.

Katz, R., Heller, S. R., Jacobson, A. E., Rotman, A., and Creveling, C. R.: Molecular orbital calculations on catecholamines - theory and experiment. Proceedings of the VII Jerusalem Symposium on Molecular and Quantum Pharmacology, in press, 1974.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Titles: 1) N-Alkylnorketobemidones.
2) Mechanism of action of Δ^8- and Δ^9-tetrahydrocannabinol from marihuana.
3) Analgesics based on the cannabinoid structure.
4) Heteroatom containing cannabinoid-like compounds.
5) 2,9β-Dimethyl-6,7-benzomorphan.

Previous Serial No.: Same

Principal Investigator: Raymond S. Wilson

Other Investigators: E. L. May, M. Rogers, A. E. Jacobson, H. J.C. Yeh, W. Klee, Candace Pert, S. Snyder, L. S. Harris, W.L. Dewey, B. R. Martin

Cooperating Units: NIMH (W. Klee), John Hopkins University (C. Pert Medical College of Va. (L. S. Harris, W. L. Dewey, B. R. Martin)

Man Years:
Total: 2
Professional: 1
Others: 1

Project Description:

Objectives: 1) Complete the synthesis of the series of N-straight-chain alkyl norketobemidones from methyl to decyl and test in vivo analgesic potency and binding affinities to an in vitro opiate receptor. If the relative analgesic potencies correlate well with the in vitro binding, this could help explain the low analgesic potencies of some of the compounds in the series.

2) Completion of the synthesis of 9-nor-Δ^8-THC, 9-nor-Δ^9-THC, and 11-methyl-Δ^8-THC and biological testing to see if hydroxylation at the 11-methyl is a prerequisite for biological activity in the THC's as has been suggested in the literature.

3) Examination of factors influencing the biological activity of 9-nor-9-hydroxyhexahydrocannabinol, a compound which has been found to have strong analgesic potency.

4) Preparation of 11-amino-Δ^8-THC and 9-nor-9-aminohexahydrocannabinol, the amino analogs of two potent hydroxy-cannabinoid analgesics.

5) Preparation and testing of 2,9β-dimethyl-6,7-benzomorphan, a nonquaternary carbon containing analgesic.

Methods Employed: Standard organic chemical procedures and instrumental techniques.

Major Findings: 1) The *in vitro* binding potencies of this series of compounds correlated well with the relative *in vivo* analgesic potencies. In addition, the relative analgesic potencies of this series of compounds correlated very well with their relative abilities to antagonize the electrically induced contractions of the guinea pig ileum.

2) 9-Nor-Δ^8-THC, 9-nor-Δ^9-THC, and 11-methyl-Δ^8-THC were synthesized and the structures were assigned based primarily on the nmr and mass spectra. 9-Nor-Δ^8-THC and 11-methyl-Δ^8-THC were tested in dogs and 9-nor-Δ^9-THC has not yet been tested. The two compounds tested showed typical cannabinoid-like effects in overt behavior and cardiovascular reactions and had potencies very similar to Δ^8-THC, but were inactive as analgesics.

3) It was found that only the equatorial 9-hydroxy isomer had analgesic activity and that the levo isomer was twice as potent as the racemic compound. The levo isomer had a potency similar to morphine and was antagonized by the narcotic antagonist naloxone. For comparison, 11-hydroxy-Δ^8-THC, the major metabolite of Δ^8-THC was prepared and also found to have morphine-like analgesic potency. 11-Methoxy-Δ^8-THC was also prepared and found to be nearly inactive as an analgesic.

4) The amino analogs have been prepared but not tested.

5) During preparation of this compound it was found that a structural misassignment was made because two protons adjacent to a ketone had exchanged with D_2O solvent before an nmr was run. This exchange was found to be catalyzed at a greater rate when a propionate salt was used as the internal standard than when the corresponding sulfonate salt was used. The final compound was found to be a weak analgesic.

Significance to Biomedical Research and the Program of the Institute:

1) The observed correlations suggest that the inactive or low potency compounds lack potency because they do not bind well to the receptor as opposed to extremely different metabolic or distribution profiles from the more active compounds. The binding correlation further substantiates that the *in vitro* binding site is related to the biological action of these narcotic analgesics.

2) The fact that 9-nor-Δ^8-THC causes cardiovascular and CNS depressant effects of the same magnitude and nature as those of Δ^8-THC is proof that metabolism to the 11-hydroxy compound is not a prerequisite for these biological actions of the THC's. 11-Methyl-Δ^8-THC was about one-half to one-third as active as Δ^8-THC, which suggests that the site of biological activity is sensitive to steric effects around the 9-methyl group.

3) This represents the introduction of a new class of non-nitrogenous analgesics with morphine-like potency. If the cannabinoid analgesics are

physically non-addicting as other cannabinoids are reported to be, this could lead to the development of therapeutically useful compounds. The antagonism of analgesia in these compounds by naloxone suggests that they may share some common mechanisms with morphine. However, these compounds do not antagonize the in vitro binding of tritiated dihydromorphine to the presumed opiate receptor indicating a different site of action.

4) The strong analgesic potency of 11-hydroxy-Δ^8-THC, the intermediate potency of Δ^8-THC, and the inactivity of 9-nor-Δ^8-THC indicates that in this instance the parent compound is inactive and the metabolite is causing the effect.

5) The observations made on the nmr spectra of intermediates in the synthesis of this compound serve as a caution to other investigators using propionate salts as internal standards in D_2O solutions. The analgesic activity of this compound provides additional evidence that a quaternary carbon is not required in narcotic analgesics.

Proposed Course: 1) Publication. 2) Examination of these compounds to see if other THC effects can be attributed to the parent compounds or just the metabolites or both. Also, it is planned to examine these compounds for anti-inflammatory activity (which has been reported for other cannabinoids) and as antagonists of prostaglandin synthetase. 3) Further examination of these compounds to see if they or their derivatives may be useful as analgesics or other CNS drugs. 4) Full range of biological testing.

Publications: Wilson, R. S. and May, E. L.: 9-Nor-Δ^8-tetrahydrocannabinol, a cannabinoid of metabolic interest. J. Med. Chem.17: 475-476, 1974.

Martin, B. R., Harris, L. S., Dewey, W. L., May, E. L., and Wilson, R. S.: Behavioral and pharmacological properties of 11-methyl- and 9-nor-Δ^8-tetrahydrocannabinol. Fed. Proc. 33: 540, 1974.

Oh-ishi, T., Jacobson, A. E., Wilson, R. S., Yeh, H. J.C., and May, E. L.: Synthesis of 2,9β-dimethyl-6,7-benzomorphan. J. Org. Chem. in press, 1974.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synthesis and Evaluation of Some O-Acylmorphines

Previous Serial No.: Same

Principal Investigators: Arthur E. Jacobson and Everette L. May

Other Investigators: None

Cooperating Units: Pharmacology unit of Medicinal Chemistry, L.C., and Department of Pharmacology, University of Michigan

Man Years:
Total: 0.5
Professional: 0.5
Other: 0

Project Description:

Objectives: To synthesize and evaluate pharmacologically, higher and lower homologs of heroin and 6-acetylmorphine for their potential as "maintenance" drugs.

Methods Employed: Usual organic chemistry techniques, hot-plate and Nilsen assays for analgesia and single-dose studies in Rhesus monkeys for physical dependence capacity.

Major Findings: 3,6-Diformylmorphine was prepared readily from morphine and an excess of formic-acetic anhydride at room temperature. The 6-formyl compound was best prepared from morphine and formic acetic anhydride at 0-25°.

3-Acetylmorphine resulted from refluxing together morphine, solid K_2CO_3, 0.9 mol. equiv. of Ac_2O and acetone. It was obtained pure as the sulfamate salt.

3,6-Dipropionylmorphine was obtained from morphine and excess propionic anhydride (100°) while 6-propionylmorphine was best prepared by adding gradually 1:1 mol. equiv. of propionic anhydride slowly (during 10 min) to a boiling solution of morphine in ethylmethyl ketone and refluxing for an additional hour.

The most analgesically active acyl compounds were 6-formyl- and 3,6-dipropionylmorphine which were more potent than heroin or the equally active 6-acetylmorphine. Curiously, 3,6-dipropionylmorphine had the shortest duration of action, 6-propionyl and 3,6-diformylmorphine had prolonged action.

Significance to Biomedical Research and the Program of the Institute: These compounds may be of possible value in maintenance programs in the event heroin clinics are begun for "hard-core" addicts.

Proposed Course: The aforementioned acyl compounds are being assessed for their capacity to substitute for morphine in an established addiction in Rhesus monkeys with the aim of finding better maintenance and detoxification narcotics. Depending on the results, higher homologs may be synthesized.

Honors and Awards: Everette L. May

1. Sixth Walter H. Hartung Memorial Lecture at the University of North Carolina School of Pharmacy, April 22, 1974.

2. Public Health Service Distinguished Service Award, April 11, 1974.

Publications: Eddy, N. B. and May, E. L.: The search for a better analgesic. Science 181: 407-414, 1973.

Ong, H. H., Oh-ishi, T. and May, E. L.: Phenylmorphan agonists-antagonists. J. Med. Chem. 17: 133-134, 1974.

Oh-ishi, T. and May, E. L.: N-Alkylnorketobemidones with strong agonist and weak antagonist properties. J. Med. Chem. 16: 1376-1378, 1973.

Oh-ishi, T., Jacobson, A. E., Wilson, R. S., Yeh, H. J.C. and May, E. L.: Synthesis of 2,9β-dimethyl-6,7-benzomorphan. J. Org. Chem. in press, 1974.

Wilson, R. S. and May, E. L.: 9-Nor-Δ^8-tetrahydrocannabinol, a cannabinoid of metabolic interest. J. Med. Chem. in press 1974.

Serial No. NIAMDD-LC-13
1. Chemistry
2. Medicinal
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Titles: 1) An alternative synthesis for 3,6-dimethyl-8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocine and the synthesis of some new 3-alkyl analogs.
2) An alternative synthesis for 5-(m-hydroxyphenyl)-2-methylmorphan and the synthesis of some new 2-alkyl analogs.
3) The synthesis, analgetic evaluation, and "receptor affinities" of N-alkyl analogs of norketobemidone.

Previous Serial No.: NIAMDD, LC, #9

Principal Investigator: Michael E. Rogers

Other Investigators: Everette L. May, Raymond S. Wilson, C. R. Creveling, and Candace Pert

Cooperating Units: Microanalytical Services & Instrumentation Section

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: 1) To develop a more practical synthesis for 3,6-dimethyl-8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocine and to prepare the 3-n-ethyl through the 3-n-heptyl analogs to be evaluated as narcotic agonists-antagonists.

2) To develop a more practical synthesis for 5-(m-hydroxyphenyl)-2-methylmorphan and to prepare the 2-n-propyl through the 2-n-heptyl analogs to be evaluated as narcotic agonists-antagonists.

3) To prepare the N-methyl through the N-decyl analogs of norketobemidone and to examine the correlation between their analgetic activities and "receptor binding affinities."

Methods Employed: Synthetic techniques, tlc, glc, mass, nmr, and ir spectroscopy.

Major Findings: 1) The 3-n-ethyl and 3-n-amyl analogs of 3,6-dimethyl-8-hydroxy-1,2,3,4,5,6-hexahydro-3-benzazocine were prepared. The analgetic potencies of these and previously prepared derivatives were found to be uninteresting. Because of recently published new syntheses of 3-benzazocines, this aspect of the project has been discontinued.

2) The original phenylmorphan synthesis of May and Murphy is afflicted by a low yield alkylation step (24%, 2-(m-methoxyphenyl)-cyclohexanone to 2-dimethylaminoethyl-2-(m-methoxyphenyl)-cyclohexanone); the low yield is due to the predominance of O-alkylation. Thus, a study was undertaken varying the conditions of solvent, base, alkylating agent and temperature in order to determine the maximal conditions for C-alkylation. By using sodium hydride and dimethyl formanide, it was possible to use dimethylaminoethyl chloride hydrochloride (thus avoiding the tedium and loss of material involved in converting this salt to the free base) as the alkylating agent improving the yield of C-alkylated material to 40%.

3) In the course of investigating alternate routes to the phenylmorphans, 3a-(m-methoxyphenyl)- and 3a—(m-hydroxyphenyl)-octahydroindole were prepared. These compounds can be viewed as semi-rigid phenylethylamines and were found to be potent inhibitors of uptake of norepinephrine in mouse heart. These amines are now being evaluated for their ability to affect norepinephrine release.

4) The in vitro binding potencies, i.e. "receptor affinities", of the N-alkyl norketobemidones correlated well with the relative in vivo analgetic potencies and with their corresponding abilities to antagonize the electrically induced contractions of the guinea pig ileum.

Significance to Biomedical Research and the Program of the Institute:

1) The benzazocines have been shown to have little promise as therapeutic agonists.

2) The improved synthesis of (-)-5-(m-hydroxyphenyl)-2-methylmorphan make this a more attractive analgetic for clinical trial.

3) The correlation between the in vitro binding potencies and in vivo analgetic potencies lend support to the hypothesis that the observed binding is to the "narcotic receptor."

4) The 3a-aryloctahydroindoles may help delimit structural and stereochemical requirements for the catecholamine uptake system.

Proposed Course: 1) To publish above findings. 2) To evaluate benzazocines as narcotic antagonists. 3) To make the 2-n-ethyl through the 2-n-hexyl-5-(m-hydroxyphenyl)-morphans and evaluate for agonist-antagonist potencies. 4) To evaluate the 3a-aryloctahydroindoles for effect on catecholamine release.

Publications: None

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Titles: 1) Synthesis of 2-(N-dansyl-N-methylaminoethyl)-derivatives of 6,7-benzomorphan.
2) Synthesis of homomorphinans.

Previous Serial No.: None

Principal Investigators: Shunsaku Shiotani (G.W.) and A. E. Jacobson

Other Investigators:

Cooperating Units: Pharmacology Unit and Section on Microanalytical Services and Instrumentation of this Laboratory

Man Years:
Total: 0.5
Professional: 0.5
Other: 0

Project Description:

Objectives: 1) To synthesize 2-(N-dansyl-N-methyl-aminoethyl)-derivatives of α-5,9-dimethyl-2'-hydroxy-6,7-benzomorphan and α-5,9-diethyl-6,7-benzomorphan and evaluate their analgesic and binding activity to putative narcotic receptors.

2) To synthesize derivatives of cis- and trans-6-methoxy-4a,10-ethanoiminomethano-1,2,3,4,4a,9,10,10a-octahydrophenanthrene and evaluate their analgesic activity.

Methods Employed: Reaction methods and techniques generally employed in organic chemistry.

Major Findings: 1) (a) Synthetic work has been completed and the benzomorphan derivatives described above were obtained in reasonable yields.
(b) Pharmacological results, - not yet available.

2) (a) The key intermediate (6-methoxy-9-oxo-4a,10-ethanoiminomethano-1,2,3,4,4a,9-10,10a-octahydrophenanthrene) for the compounds described above has been synthesized by standard methods.

Significance to Biomedical Research and the Program of the Institute: Constitutes a part of the overall research effort toward a chemicopharmacological solution to the problem of narcotic addiction and toward the search for improved substitutes for morphine and codeine.

Proposed Course: To continue two. The final objective compounds must be synthesized.

Publications: None

PHS-NIH
Individual Project Report
September 1973 through June 30, 1974

Project Title: Pharmacologic evaluation of experimental compounds, potential analgesics.

Previous Serial No.: None

Principal Investigator: Ludmilla Syrotenko-Getsiv

Other Investigators: None

Cooperating Units: Instrumentation Services

Man Years:
Total: 0.5
Professional: 0.5
Others: 0

Project Description:

Objectives: 1) Familiarization with activities, objectives and methodology of the Section. 2) In-depth literature search in areas of particular relevance to the research objectives of the Section. 3) Laboratory investigation of newly synthesized compounds. 4) Development of the long range research proposal considering the investigative methodology and research interests of the Section.

Methods Employed: 1) Briefings, discussions, research publications by the members of the Section. Observation of laboratory procedures, employed in the Section, for the determination of potential analgesic activity and the determination of ED_{50} by the computerized probit analyses. 2) Systematic reviews of literature from the intra-section circulation of current issues, 101 publications in December-March period, and basic NIH library collection. 3) The Nilsen method was used for the pharmacologic investigation of the NIH 8933 and NIH 8950 compounds. 4) Preliminary investigative work to support the theoretical justification of the long range research proposal and accumulation of information for the assessment of economic feasibility.

Major Findings: NIH 8933 was found to be toxic, in a short study of acute effect, and was not investigated further. NIH 8950 was examined for acute and chronic effects. It exhibited some unexpected physiologic effects warranting further investigation but the study was temporarily suspended in

anticipation of the availability of an additional quantities of the compound. To establish the basis for the chronic investigation of the current and future compounds, the control study of normal population of mice was initiated, and is being continued. Modification of the pre shock conditions (Nilsen) resulted in a 25% increase in the number of experimentally acceptable mice. In order to develop a greater degree of quantitation and precision with the Nilsen method, the voltage stimulator design was diversified, in cooperation with the NIH Biomedical Engineering and Instrumentation Branch; the new model giving very satisfactory results. The method will be further improved by the use of a modified mouse-holder assembly and addition of the audio and kinetic components to the stimulator system, all in the process of being executed by the Biomedical Engineering and Instrumentation Branch.

Significance to Biomedical Research and the Program of the Institute: It is of interest to evaluate with precision medicinally useful potent analgesics and to progress toward deeper understanding of the chemical basis for their physiologic effects.

Proposed Course: Research for the immediate future is visualized as encompassing work with a short and a long range goal in mind, the two proceeding concurrrently. The former, would involve in-depth pharmacologic study of compounds of current interest and the latter a systematic investigation of the role, or lack of it, of brain catecholamines in the mechanism of action of morphine. The tentative working title: Brain Catecholamines and the Antinociceptive Effect of Morphine.

Publications: None

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synthesis of 2'-hydroxy-9-methyl-6,7-benzomorphans and their derivatives.

Previous Serial No.: NIAMDD, LC, #15

Principal Investigator: Hirozumi Inoue (Visiting Fellow) and E. L. May

Other Investigators: None

Cooperating Units: Pharmacology unit and Microanalytical Services & Instrumentation Section

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: To synthesize 2'-hydroxy-9-methyl-6,7-benzomorphans and derivatives by known or new routes and evaluate their analgesic activity.

Methods Employed: Reaction methods and techniques generally employed in organic chemistry.

Major Findings: A possible and improved synthetic routes to the long sought 2'-hydroxy-9-methyl-6,7-benzomorphans lacking an alkyl substituent at C-5 was developed and the β-isomer of 2'-hydroxy-2,9-dimethyl-6,7-benzomorphan was synthesized in 5.8% overall yield from m-methoxyphenylacetone via the 3,5-cis isomer of 4-(2-dimethylamino)ethyl-3-methyl-1,2,3,4-tetrahydro-1-naphthalenone.

Application of the reaction of 5-dimethylamino-3-(m-methoxyphenyl)-2-pentanone with lithio ethyl acetate instead of the Reformatsky reaction with bromo-ethyl acetate gave the desired product in quantitative yield.

Diastereoisomers of 4-(2-dimethylamino)ethyl-3-methyl-3,4-dihydro-1(H)-naphthalenone were isolated and the stereo chemical structures were determined

by investigation of NMR spectrum of these compounds (and then bromo derivatives) and by their behavior toward bromination and cyclization.

The other difficult step due to substitution of 2'-methoxy group was reduction of 8-oxo group of 2'-methoxy-8-oxo-2,9-dimethyl-6,7-benzomorphan to methylene. While ordinary W. K. reduction resulted in very low yield, hydrogenation of 8-oxo derivatives in the presence of Pd-C and $HClO_4$ in HOAc under low pressure and reduction of 8-hydroxy derivative with P and HI in HOAc gave good results.

Bromination of the 3,4-trans-isomer of the above naphthalenone and then cyclization with NH_4OH did not give the benzomorphan skeleton but 6-methoxy-4(2-dimethylamino)ethyl-3-methyl-1-naphthol as in the 6-demethoxy series.

Cyclization of 1-cyano and 1-ethoxycarbonyl derivatives of 2-(p-methoxybenzyl)-3-methyl 1,2,5,6-tetrahydropyridine in many kinds of reaction conditions did not give the desired 9-methyl-benzomorphan.

The other steps of demethoxy series were applicable for this methoxy series.

2,9β-Dimethyl-2'-hydroxy-6,7-benzomorphan is comparable to morphine in analgesic activity while the deoxy analog is codeine-like with higher toxicity. These are the first non-quaternary-carbon 9β-alkylbenzomorphans to be obtained.

Proposed Course: The α-isomer of 2'-hydroxy-9-methyl-6,7-benzomorphan must be synthesized and a short step method of this benzomorphans must be established.

Publications: None

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synthesis and Biochemistry of Potential Antiviral and Carcinostatic Nucleotides and Nucleosides

Previous Serial Number: NIAMDD-LC-18

Principal Investigator: Paul F. Torrence and James A. Waters

Other Investigator: B. Witkop

Cooperating Units: Florence White, NCI-DE

Man Years:

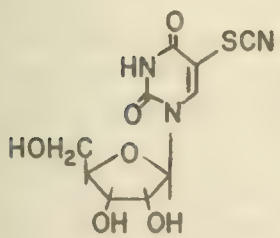
Total: .2
Professional: .2
Others: 0

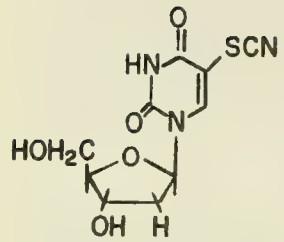
Project Description:

Objectives: The aims of this project are to investigate the chemistry and biochemistry of both normal and "fraudulent" nucleosides with the objective of developing basic knowledge leading to effective anti-viral and anti-cancer agents at the monomer (nucleoside) and polymer (polynucleotide) levels.

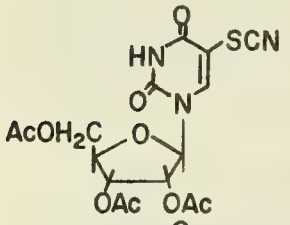
Methods Employed: Thin-layer, paper, ion-exchange and column chromatography, liquid scintillation, ultraviolet, visible, infrared, nuclear magnetic resonance, circularr dichroism and mass spectroscopy.

Major Findings: The biological activity of the previously reported 5-thiocyantouracil derived nucleosides has been evaluated in several cell lines.

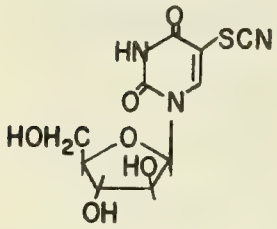
nucleoside	concentration causing 50% inhibition of growth in		
	KB cells	L5178Y	Vaccinia virus replication in HeLa cells
	$2x10^{-4}$ M	$5x10^{-5}$ M	--



9×10^{-6} M 8×10^{-7} M 1×10^{-4} M



$>3\times10^{-4}$ M $>1\times10^{-4}$ M --



-- $>1\times10^{-4}$ M --

It was shown that the thiocyanate analogs could be easily reduced with glutathione to the corresponding 5-mercapti derivatives. They are probably also reduced in vivo to the mercapto derivatives, already known to be active.

Significance to Biomedical Research and the Program of the Institute: The synthesis and reduction of 5-thiocyanate pyrimidine nucleosides provides a quick convenient method of synthesizing potential antimetabolites which may be useful in understanding cellular processes or in the design of useful antiviral or anti-tumor agents.

Proposed Course: This general problem has diversified so much that this title is to be terminated as such, but outgrowths of this work are to be found in a number of new reports accompanying this one.

Honors and Awards: None

Publications:

Torrence, P., Bobst, A., Waters, J. and Witkop, B., "Synthesis and Characterization of Potential Interferon Inducers: Poly(2'-azido-2'-deoxyuridylic acid)", Biochemistry 12, 3962-3972 (1973).

Torrence, P., Waters, J. A., Buckler, C. E. and Witkop, B., "Effect of Pyrimidine and Ribose Modifications on the Antiviral Activity of Synthetic Polynucleotides", Biochem. Biophys. Res. Comm., 52, 890-898 (1973).

Nagamachi, T., Fourrey, J. L., Torrence, P. F., Waters, J. A., and Witkop, B., "Synthesis, Chemistry and Biological Activity of 5-Thiocyanatopyrimidine Nucleosides as Potential Masked Thiol". J. Med. Chem. 17, 403-406 (1974).

Serial No. NIAMDD-LC-18
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Role of Strandedness in the Induction of Interferon by Synthetic Polynucleotides

Previous Serial Number: NIAMDD-LC-19

Principal Investigator: Paul F. Torrence

Other Investigator: B. Witkop

Cooperating Units: E. De Clercq, Rega Institute for Medical Research, Leuven, Belgium

Man Years:

Total: .2
Professional: .2
Others: 0

Project Description:

Objectives: As explained in our previous report, we wished to ascertain the importance, if any, of the rearrangement of poly(A)·poly(U) to poly(A)· 2 poly(U) in determining the relative effectiveness (compared to poly(I)·poly(C)) of poly(A)·poly(U).

Methods Employed: Thin-layer, paper, ion-exchange and column chromatotraphy, liquid scintillation, ultraviolet, visible, infrared, nuclear magnetic resonance, circular dichroism, mass spectroscopy, ultracentrifugation and tissue culture.

Major Findings: The synthesis, characterization and biological evaluation of a number of polyadenylic acid and poly(7-deazaadenylic acid) complexes provided the following information regarding the interferon induction process.

a) The inactivity of certain double-stranded polynucleotides as interferon inducers can be related to their conformation and thus to their lack of recognition by a cellular receptor site.

b) The inactivity of several polyadenylic acid complexes as interferon inducers can be related to a physical rearrangement which can be prevented. This led to an inducer, poly(A)·poly(rT), which is as active as the well known poly(I)·poly(C).

c) Single-stranded polynucleotides are poor interferon inducers because they do not interact with the cellular receptor site for interferon inducers.

Inactive double-strands and triple-strands interact with the receptor site, but do not turn on interferon because they may not possess a proper conformation for triggering the necessary message for interferon production.

Significance to Biomedical Research and the Program of the Institute: These findings represent a major advance in our understanding of factors involved interferon induction by synthetic polynucleotides and pave the way for the design of inducers more active than poly(I)·poly(C) in the therapy of viral and/or neoplastic disease.

Proposed Course: To learn more of the above factors other polynucleotides systems are being investigated.

Honors and Awards: None

Publications:

E. De Clercq, P. F. Torrence and B. Witkop, "Interferon Induction by Synthetic Polynucleotides. Importance of Purine N-7 and Strandwise Rearrangement", Proc. Nat. Acad. Sci. USA 71, 182-186 (1974).

E. De Clercq, P. F. Torrence, B. Witkop and P. De Somer, "Interferon Induction by Synthetic Polynucleotides, Competition between Inactive and Active Inducers", Proceedings of the Gulbenkian Meeting on Interferon, Oerias, Portugal, Sept. 1973, C. Chancy, ed., Academic Press, N. Y. in press.

Se[illegible] NIAMDD-LC-19
1. [illegible]istry
2. [illegible]olites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Photoinduced Reaction of Uridine with Cyanide Ion.

Previous Serial Number: NIAMDD-LC-20

Principal Investigator: J. A. Waters

Other Investigators: B. Witkop

Cooperating Units: None

Man Years:

Total: .2
Professional: .2
Others: 0

Project Description:

Objectives: To modify nucleosides by photochemical processes and to evaluate the photoproducts for antitumor or antiviral activity.

Methods Employed: An aqueous solution of uridine (0.02 M) in the presence of potassium cyanide (0.2 M) was irradiated with a low-pressure mercury lamp (254 nm) from 36 to 66 hr.

Major Findings: A single compound is formed in high yield (>90%). Analytical and spectral data have shown the product to be dihydroorotidine, potassium salt. In this novel reaction, the primary photoprocess is attack of cyanide at the 6-position, with the adduct undergoing a secondary photoreaction in basic media to produce the nucleoside carboxylic acid (DHO).

Significance to Biomedical Research and the Program of the Institute: Modifications of the pyrimidine ring at the 5- and 6-positions have produced clinically useful nucleosides.

Proposed Course: To isolate the cyano intermediate, to complete the mechanistic studies and to submit the compounds for testing.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-20
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Photochemical and Anodic Decarboxylations of Orotidine

Previous Serial No.: NIAMDD-LC-21

Principal Investigator: J. A. Waters

Other Investigator: B. Witkop

Cooperating Units: None

Man Years:

Total: .2
Professional: .2
Others: 0

Project Description:

Objectives: To determine whether photochemical and anodic decarboxylations of orotidine and OMP could serve as models for the enzymatic decarboxylation of OMP to UMP by OMP decarboxylase.

Methods Employed: (A) Aqueous solutions of orotidine and OMP were photolyzed with a low pressure mercury lamp (254 nm). (B) Orotidine, cyclohexyl ammonium salt, at basic pH was electrolyzed using platinum electrodes (100 ma., 10 v.)

Major Findings: (A) Kinetics (uv and chromatography) show the complete disappearance of O and OMP in 18 hr. and 16 hr, respectively. The major products from these reactions were U (uv, nmr, tlc) and UMP (uv, paper chromatography). (B) Uridine and two other products are formed. The anodic reaction is not as efficient as the photoprocess.

Significance to Biomedical Research and the Program of the Institute: Since certain mechanistic implications of these in vitro decarboxylations are established, they may aid in a more complete understanding of the biosynthetic process.

Proposed Course: To elucidate the reaction mechanism of the photoreaction by quenching and other experiments.

Honors and Awards: None

Publications: None

Serial No NIAMDD-LC- 21
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synthetic Polynucleotides as Potential Inducers of Interferon

Previous Serial Number: NIAMDD-LC-22

Principal Investigator: J. A. Waters and P. F. Torrence

Other Investigator: B. Witkop

Cooperating Units: E. De Clercq, Katholieke Universiteit Te Leuven, Leuven, Belgium

Man Years:

Total: .4
Professional: .4
Other: 0

Project Description:

Objectives: To synthesize various base-modified polynucleotides for evaluation as inducers of interferon.

Methods Employed: The 5'-diphosphates of 7-deazainosine and formycin B were synthesized and then polymerized by polynucleotide phosphorylase. Physical measurements were made on the homopolymers and their complexes with poly(C) and poly(br^5C). Interferon titers were obtained using primary rabbit kidney cells and activity/toxicity ratios in mouse L-929 cells.

Major Findings: Poly(c^7I)·poly(br^5C) was twice as active as poly(I)·poly(C) in interferon primed PRK cell cultures and ten times more active (0.1 g/ml dose) in those cells superinduced with cycloheximide and actinomycin D. The activity/toxicity ratio of poly(c^7I)·poly(br^5C) was greater than that of poly(I)·poly(C) at several doses. Poly(formycin B) complexes were inactive in PRK cells.

Significance to Biomedical Research and the Program of the Institute: Modifications of the standard synthetic polynucleotides may provide an efficient, non-toxic inducer of interferon in humans and may provide insight for future synthetic approaches to antiviral-antitumor drugs. The poly(c^7I)·poly(br^5C) complex is the most active interferon inducer with a high therapeutic index known thus far.

Proposed Course: The activity/toxicity ratio of poly(c^7I)·poly(br^5C) is being determined in animals. The syntheses of poly(2-aza I) and poly(7-bromo-7-deaza I) are in progress.

Honors and Awards: None.

Publications: None

Serial No. NIAMDD-LC-22
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Photocyclizations

Previous Serial Number: NIAMDD-LC-23

Principal Investigator: Bernhard Witkop

Other Investigators: None

Cooperating Units: O. Yonemitsu, Y. Kanaoka and T. Iwakuma, University of Hokkaido, Sapporo, Japan
N. Kanamaru and K. Kimura, Research Institute of Applied Electricity, Hokkaido University, Sapporo, Japan
I. L. Karle, U. S. Naval Research Lab., Washington, D.C.

Man Years:

Total: 1.0
Professional: .5
Others: .5

Project Description:

Objectives: Reactions for the selective modification of simple amino acids in proteins are needed for the study of the composition and function of their active sites. Although extensive interest to develop such reactions has been shown by many investigators, photochemical methods were applied only recently to this problem. Photochemical methods have been elaborated which make it possible to deliver selectively a distinct amount of energy to a system. The photochemical behavior of free and bound aromatic amino acids and the corresponding pharmacodynamic amines and certain of their derivatives therefore have been investigated.

Methods Employed: Ultraviolet irradiation, flash photolysis thin-layer chromatography, silica gel and ion exchange column chromatography, paper chromatography and electrophoresis, UV, IR, NMR and mass spectroscopy.

Major Findings: 1. Photocyclization of N-Alicyclic Phthalimides:Synthesis of Multicyclic Benzazepine Systems. N-Alkylated phthalimides generally undergo photocyclization to cyclobutanols in a Norrish type II photoprocess followed by retro-transannular ring opening to benzazepinones. When the 4-alkyl substituent has γ-hydrogen with respect to the newly formed ketonic carbonyl (position 5), type II photoelimination occurs with 4-dealkylation.

Irradiation of N-cyclopentylphthalimide in 10 mM acetonitrile solution with a 1 kw high-pressure mercury lamp for 2 hr afforded the 3,4-cyclobutanobenzazepinone in 48% yield whose 3,4-cis-junction was confirmed by independent synthesis from 3-ethoxyisoindolone and cyclopentene. Likewise, N-cyclohexylphthalimide afforded, as expected, the homologous benzazepinone whose structure was established by direct comparison with an authentic sample. In addition a new compound, mp 244-246°, was isolated whose yield on photolysis in t-butanol increased to 23%, while N-cyclohexylphthalimide under the same conditions afforded exclusively another novel product in 55% yield, of which an X-ray diffraction analysis of a single crystal established the complete structure as 1,2,2a,3,4,9b-hexahydro-9b-acetoxy-9b-acetoxy-1,3-propano-5H-cyclobuta-[d][2]benzazepin-5-one.

2. Oxidative and Nonoxidative Pathways in the Photocyclization of Five-Membered Heterocyclic Anilides. Heterocyclic anilides, such as thiophene-2-and indole-2-carboxanilide, undergo photocyclication in the presence of dissolved oxygen to afford the heterocyclic condensed quinolones. Whereas oxidative cyclizations of anilide systems are closely related to the well-known photoreactions in the stilbene series, Chapman, et al. established nonoxidative photocyclization of N-arylenamines.

On irradiation in a mixture of benzene and ethanol (10 :1 v/v; 200 mg/200 ml) with a 100 W high-pressure mercury lamp for 2 hr, thiophene-2-carboxanilide cyclized oxidatively to 3,4(a,b)thiophenoquinolone-2 in 45% yield, accompanied by a crystalline product A with the same composition as the starting material B. When the reaction was performed under a stream of nitrogen in a solvent free of oxygen, A was obtained as the major product (50%) along with a trace of B. Although A was converted into B by iodine oxidation, it is unlikely to be the true intermediate of the transformation because on irradiation under aerobic conditions A afforded B but with much lower efficiency. Thus A must be the product arising from a plausible intermediate, as a result of a process that competes with the direct oxidation to B. The complete structure of A was directly established by X-ray analysis as trans-3,4-dihydro-3,4(a,b) thiophenoquinolone-2.

Significance to Biomedical Research and the Program of the Institute: New approaches to novel heterocyclic systems of potential CNS activity.

Proposed Course: Extension into systems relating to pharmacodynamic amines.

Honors and Awards: none

Publications:

Kanaoka, Y., Koyama, K., Flippen, J. L., Karle, I. L., Witkop, B., Photocyclizations of N-Alicyclic Phthalimides. Synthesis of Multicyclic Benzazepine Systems, JACS, in press.

Iwakuma, T., Nakai, H., Yonemitsu, O. and Witkop, B.: Photocyclizations of N-Chloroacetyltyramine. I. Formation of Novel Dimeric Cage Compounds. J. Amer. Chem. Soc. 96, 2564-2569 (1974).

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Batrachotoxin, Pumiliotoxin, Histrionicotoxin and Other Physiologically Active Compounds from Neotropical Frogs.

Previous Serial Number: NIAMDD-LC-24

Principal Investigator: Bernhard Witkop

Other Investigators: J. Daly, G. Brown, and W. Kissing, E. Gössinger

Cooperating Units: Charles W. Myers, American Museum of Natural History, New York, N. Y.
E. X. Albuquerque, Dept. of Pharmacology, University of New York at Buffalo, N. Y.
Isabella Karle, U. S. Naval Research Laboratory, Washington, D. C.
G. Habermehl, Technische Hochschule, Darmstadt, Germany
T. Tokuyama, Osaka City University, Osaka, Japan
E. J. Corey, Harvard University
H. Wehrli, ETH, Zurich, Switzerland
E. Bartels de Bernal, Universidad de Valle, Cali, Columbia
J. Gardner, D and HD: NIAMDD

Man Years:

Total: 1.0
Professional: .5
Other: .5

Project Description:

Objectives: To elucidate the molecular basis for the pharmacological activity of batrachotoxin, tetrodotoxin, histrionicotoxin and related substances. To isolate and elucidate the structures of other pharmacodynamically active substances found in skin extracts of neotropical frogs. To explore biosynthetic pathways involved in the formation of these unique substances.

Methods Employed: Sensitive bioassay methods, thin layer, high pressure liquid, column and gas chromatography have been used in the purification of the active principles, while NMR, mass spectroscopy, x-ray crystallography, microchemical techniques and radiochemical labeling are being utilized for structure elucidation. Isotopic tracer techniques have been used to study biosynthetic pathways. Biological activity has been investigated with both isolated enzyme systems and in intact cells.

Major Findings: Investigation of the unique pharmacological properties of batrachotoxin and various analogs has continued. In rat diaphragm preparations, batrachotoxin elicits an increase in spontaneous transmitter release at 28° only in the presence of calcium ions. At 37°, the presence of calcium is not essential to batrachotoxin-elicited transmitter release. The results are compatible with the proposal that under calcium-free conditions, transmitter release depends on an internal calcium pool which can be mobilized at a critical temperature of $> 30^{\circ}$ by batrachotoxin-elicited increases in internal sodium concentration. All analogs of batrachotoxin cause membrane depolarization by eliciting an increase in Na^{+} conductance, but their relative potencies differ markedly in various biological preparations. Indeed, in mammalian neuromuscular preparations, certain analogs preferentially affect presynaptic membranes, while others affect the postsynaptic muscle membrane. In cardiac preparations, batrachotoxin and the various analogs all elicited positive inotropic effects, membrane depolarization and finally arrhythmias. Radioactive batrachotoxin has been prepared and shown to bind irreversibly to a small number of sites in mouse diaphragm preparations.

The unique properties of histrionicotoxin and various analogs have been investigated further. The effects of atropine, diisopropylfluorophosphate, and histrionicotoxin on neuromuscular transmission are most easily rationalized in terms of interactions with an ion-conductance modulator protein rather than with the associated acetylcholine receptor or with acetylcholine esterase. The action of histrionicotoxin appears due to combination with the ion conductance modulator in its open conformation, thereby leading to a suppression of the endplate conductance. The interaction is strongly dependent on the membrane potential. Of the various analogs, dihydroisohistrionicotoxin has the highest affinity for this site. Histrionicotoxin, conversely is the most active compound of the series with respect to the potassium channel involved in action potential generation. A third compound, octahydrohistrionicotoxin is a very active local anaesthetic which may block both sodium and potassium conductances.

Synthesis of Histrionicotoxin without Side Chains: Starting with N-hydroxypiperidine, oxidation to the mixture, Grignard reaction with 4,5-dehydroamylmagnesium bromide, re-oxidation to the nitron and thermal cyclization and equilibration Dr. E. Gössinger obtained 1,7-oxido-azaspiro[5,5]undecane which on hydrogenolysis liberatet the 7-hydroxyl in the correct position. This compound had none of the activity characteristic of histrionicotoxin. Starting with ethyl cyclopentanone-2-carboxylate, 2-alkylation, and Schmidt-rearrangement Dieckmann cyclization Dr. W. Kissing obtained 2,7-dioxo-1-azaspiro[5,5]undecanes.

Significance to Biomedical Research and the Program of the Institute: The exceptionally high toxicity of batrachotoxin, its selective action on nerve preparations and its cardiotoxin properties gave the elucidation of its structure and correlations of structures with activity a high priority in biomedical research. Because of their remarkable effects on ion conductances, histrionicotoxin and various analogs provide unique tools for the study of the acetylcholine modulated ion channels in electrogenic membrane. Other compounds from frog extracts also have physiological activities which warrant their structural and pharmacological investigation. Examples of such compounds occurring in frogs and toads include the pumiliotoxins, samandarine, the bufogenins, the catecholamines, indolealkylamines and histamines, and recently hypotensive polypeptides such as bradykinin and physalaemin. Because of its structural similarity to bufotenine and lysergic acid diethylamide, a thorough investigation of the properties of dehydrobufotenine is relevant to fundamental aspects of neurochemistry.

Proposed Course of Project: The structures of various alkaloids from frogs of the genera *Dendrobates*, *Colosthetus* and *Atelopus* will be elucidated. The pharmacological activity of batrachotoxin and histrionicotoxin analogs will be studied in detail. Radioactive batrachotoxin, tetrodotoxin and histrionicotoxin will be prepared and used to study their ultra-structural site of action. The unique oxidative cyclization of indolealkylamines to dehydrobufotenine will be investigated and attempts will be made to isolate the enzyme system responsible. Further isolations of batrachotoxins, histrionicotoxins, and pumiliotoxins will be carried out to provide material for chemical and pharmacological study.

Honors and Awards:

Publications:

Tokuyama, T., Uenoyama, K., Brown, G., Daly, J. W., and Witkop, B.: Allenic and acetylenic spiropiperidine alkaloids from the neotropical frog, *Dendrobates Histrionicus*. Helv. Chim. Acta. In press.

Albuquerque, E. X., Kuba, K., and Daly, J.: Effect of histrionicotoxin on the ionic conductance modulator of the cholinergic receptor: A quantitative analysis of the endplate current. J. Pharmacol. Exptl. Therap. In Press.

Kuba, K. Albuquerque, E. X., Daly, J., and Barnard, E. A.: A study of the irreversible cholinesterase inhibitor, diisopropylfluorophosphate on time course of endplate currents in frog sartorius muscle. J. Pharmacol. Exptl. Therap. In press.

Jansson, S.-E., Albuquerque, E. X., and Daly, J.: The pharmacology of batrachotoxin. VI. Effects on the Mammalian motor nerve terminal. J. Pharmacol. Exptl. Therap. In press.

Lapa, A. J., Albuquerque, E. X., and Daly, J.: An electrophysiological study of the effects of d-tubocurarine, atropine and α-bungarotoxin. The cholinergic receptor in innervated and chronically denervated mammalian skeletal muscle. Experimental Neurology. In press.

Albuquerque, E. X., Kuba, K., Lapa, A. J., Daly, J., and Witkop, B.: Acetylcholine recpetor and ionic conductance modulator of innervated and denervated muscle membranes. Effect of histrionicotoxin. Proc. Int. Conf. of the Muscular Dystrophy Assoc. of American, Oct. 14-19, 1973, Excerpta Medica. In press.

Witkop, B.: Natural toxins as originators of progress in neurophysiology. La Recherche. In press.

Brown, G., and Witkop, B.: Natural toxins as tools in neurophysiology. Israel Journal of Chemistry. In press.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Interactions of Polynucleotides at the Cellular Level. Use of the Interferon System to Identify New Polynucleotide Complexes or Displacement Reactions.

Previous Serial Number: None

Principal Investigator: Paul F. Torrence

Other Investigator: B. Witkop

Cooperating Units: E. De Clercq, W. E. Stewart II, P. De Somer, Rega Institute for Medical Research, University of Leuven, Belgium

Man Years:

Total: .2
Professional: .2
Others: 0

Project Description:

Objectives: To determine if polynucleotide interactions occurring normally in solution also occur at the cellular level. To use the interferon system as a method of screening for new or unexpected polynucleotide interactions.

Methods Employed: Temperature-dependent ultraviolet spectriscopy, circular dichioism, liquid scintillation, paper and column chromatography, equilibrium dialysis, tissue culture.

Major Findings: It has been demonstrated that the following polynucleotide reactions occur at both the cellular level and in the test-tube in vitro.
2 poly(C) + poly(A)·2 poly(I)→2 poly(I)·poly(C) + poly(A)
poly(A)·poly(U) + poly(dUz)→poly(A)·poly(U)·poly(dUz)
poly(A)·poly(rT) + poly(dUz)→poly(A)·poly(rT)·poly(dUz)
poly(I) + poly(A)·poly(rT)→poly(A)·poly(rT)·poly(I)
poly(I) + poly(A)·poly(U)→poly(A)·poly(U)·poly(I)
The last two reactions represent a previously unrecognized polynucleotide interaction; namely, the poly(A)·poly(U)·poly(I) triplex. This is the first example of a triple-stranded complex involving three different heterocyclic bases and may have significant implications as regards the secondary and tertiary structure of RNA and DNA.

Significance to Biomedical Research and the Program of the Institute: There studies will lead to a increased understanding of factors important in determining structure and stability in nucleic acids. They may define potential interactions of importance in such areas of regulation of gene expression. Interactions of polynucleotides at the cellular level should lead to a clearer understanding of what happens in the induction of interferon by synthetic polynucleotides.

Proposed Course: The above investigations will be continued and expanded to other polynucleotides especially those related to possible modes of gene regulation and nucleic acid structure.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-25
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Polynucleotide Inhibition of the Reverse Transcriptase

Previous Serial Number: None

Principal Investigator: Paul F. Torrence, James A. Waters

Other Investigator: B. Witkop

Cooperating Units: E. De Clercq, Rega Institute for Medical Research
Leuven, Belgium
F. Eckstein, Max Planck Institute Göttingen, Germany
B. Janik, Miles Laboratories, Elkhart, Indiana

Man Years:

Total: .2
Professional: .2
Others: 0

Project Description:

Objectives: To determine if modified polynucleotides can serve as effective inhibitors of RNA-instructed DNA polymerase (RIDP).

Methods Employed: Liquid scintillation counting, tissue culture.

Major Findings: Poly(2'-azido-2'-deoxyuridylic acid) (Poly(dUz)) is a more potent inhibitor of the RIDP from Maloney Murine Leukemia Virus than is poly(U) or other 2'-modified polynucleotides. Poly(ribothymidylic acid) and poly(7-deazainosinic acid) do not inhibit this enzyme.

Significance to Biomedical Research and the Program of the Institute: Since RIDP is believed to play a role in the malignant transformation of cells by some oncogenic virus, inhibitors of this enzyme are of obvious interest.

Proposed Course: Other modified polynucleotides will be examined for their effect on RIDP. Currently we are determining whether or not poly(dUz) can block malignant transformation *in vitro* and *in vivo*.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-26
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: 2,4,5-Trimethylpyrrole-3-carboxylic Acid Esters of Various Tropanols as Potential Analgesics

Previous Serial No: None

Principal Investigator: J. A. Waters

Other Investigator: B. Witkop

Cooperating Units: E. L. May, LC, NIAMDD

Man Years:

Total: .2
Professional: .2
Other: 0

Project Description:

Objectives: To synthesize 2,4,5-trimethylpyrrole-3-carboxylic acid esters of tropanols related to scopoline (3α,6α-epoxy-7β-hydroxytropane) and to assay them for analgesic activity.

Methods Employed: Various tropanols, and partial structures thereof, have been esterified in high yield under mild conditions with trifluoroacetic acid-pyrrole carboxylic acid mixed anhydride.

Major Findings: Tropanols thus far successfully esterified are 3α-tropanol (tropine) and 3β-tropanol (pseudotropine). Partial structures esterified are 3-hydroxy-N-methylpiperidine, 4-hydroxy-N-methylpiperdine and 3-hydroxy-N-methylpyrrolidine.

Significance to Biomedical Research and the Program of the Institute: The previously synthesized scopoline ester was found to possess analgesic activity in the hot plate assay (20% more active than codeine) and the Nilsen assay (one-sixth that of codeine). Compounds now being synthesized may possess greater potency and provide useful structure-activity relationships.

Proposed Course: To synthesize several more candidates and evaluate them for analgesic activity.

Honors and Awards: None

Publications: J. A. Waters, C. R. Creveling and B. Witkop, "2,4,5-Trimethylpyrrole-3-carboxylic Acid Esters of Various Alkaloids", J. Med. Chem., in press.

Serial No. NIAMDD-LC-27
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synthesis of Depot Forms of Biologically Active Nucleosides

Previous Serial Number: None

Principal Investigator: J. A. Waters

Other Investigator: B. Witkop

Cooperating Units: None

Man Years:

Total: .2
Professional: .2
Other: 0

Project Description:

Objectives: To potentiate the therapeutic activity of biologically active nucleosides by selective esterification with heteroaromatic acids.

Methods Employed: Selected nucleoside are being esterified with heteroaromatic acids, including 2,4,5-trimethylpyrrole-3-carboxylic acid, using a modification of the TFAA mixed anhydride procedure developed in this laboratory.

Major Findings: Uridine and 2',3'-isopropylideneuridine were initially esterified to determine the scope of the reaction. The ester of the biologically active 5'-fluoro-2'-deoxyuridine (FUDR) has been obtained in high yield.

Significance to the Biomedical Research and the Program of the Institute: These esters of active nucleosides should act as molecular depot forms (cf. adamantoylation: Neil, 1970; Wechter, 1971) by effecting the rate of dissolution, lipophilicity and susceptibility to catabolic conversion of the active nucleoside.

Proposed Course: To synthesize esters of cytosine arabinoside (ara C), pyrazomycin, and others and to evaluate them for antileukemic, antiviral and immunosuppressive activity.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LC-28
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Modification of Free and Bound Tryptophan.

Previous Serial Number: NIAMDD-LC-17

Principal Investigator: Thomas F. Spande

Other Investigators: B. Witkop

Cooperating Units: M. Ohno, Kyushu University, Fukuoka, Japan

Man Years:

Total: .5
Professonal: .5
Other: 0

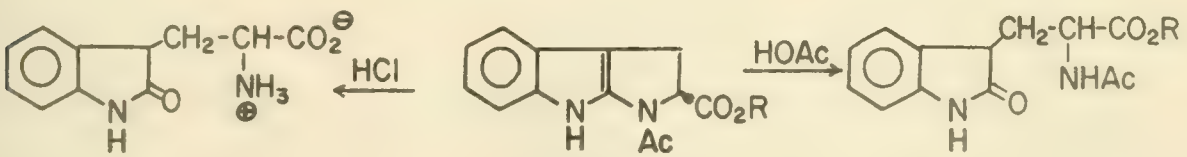
Project Description:

Objectives: To study the chemical modification of indole models, tryptophan derivatives and tryptophan in peptides and proteins as a means of assessing the role of tryptophan residues in the structure and function of proteins. To prepare and study various oxidized derivatives of tryptophan.

Methods Employed: UV, IR, and NMR Spectroscopy, mass spectrometry, thin layer and column chromatography.

Major Findings: A high-yield, convenient new synthesis of L-2-hydroxy-tryptophan (*B*) of high optical purity has been achieved by acidic (6NHCl) hydrolysis of the pyrroloindole (*A*), produced in turn by the hypochlorite oxidation of N-acetyl-L-tryptophan esters.

When 20% acetic acid is used in the hydrolysis of *A*, N-acyl esters (*C*) of *B* result in 63(72=CH_3) or 75% (R=C_2H_5) yield.



B A C

R = CH_3, C_2H_5

Significance to Biomedical Research and the Program of the Institute:

L-2-hydroxytryptophan is a naturally occurring precursor of insect eye pigments, and a hydrolysis product of a number of toxic mushroom cyclic peptides. A convenient synthesis of pure L-material may be of interest to investigators in these areas as well as to possible studies on peptides of L-2-hydroxytryptophan.

Proposed Course: To study the reactions of *A* under other conditions of solvolysis, *e.g.* reactions with NaOEt-EtOH, anhydrous HOAc, ROH, RSH etc. as routes to other tryptophan derivatives.

Honors and Awards: None

Publications: M. Ohno, T. F. Spande and B. Witkop, "A New, Practical Synthesis of L-2-Hydroxytryptophan and Its Derivatives", J. Org. Chem., in press, 1974.

Serial No. NIAMDD-LC-29
1. Chemistry
2. Metabolites
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synthesis and Elucidation of Natural Products

Previous Serial Number: None

Principal Investigator: Thomas F. Spande

Other Investigators: B. Witkop, H. J. Yeh

Cooperating Units: M. Potter, S. Rudikoff, Laboratory of Cell Biology, N.C.I. D. Davies, E. Padlan, D. Segal, Laboratory of Molecular Biology, NIAMDD. R. Kuttan, N. G. Nair, A. N. Radhakrishnan, Wellcome Research Unit, Tamil Nadu, India.

Man Years:

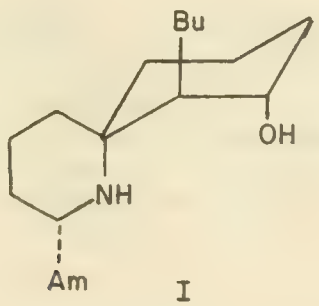
Total: .5
Professional: .5
Others: 0

Project Description:

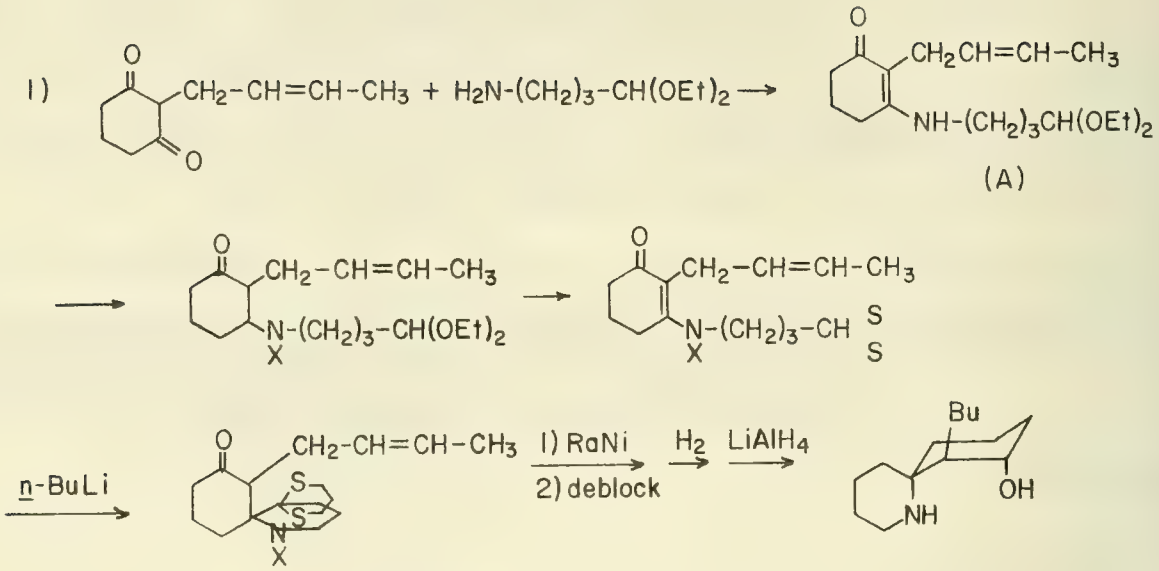
Objectives: To elucidate the structures of natural products from sandal, to develop synthetic routes to perhydrohistrionicotoxin, to develop a heavy atom containing hapten for a phosphoryl choline binding in a myeloma protein.

Methods Employed: UV, IR and NMR spectroscopy, mass spectrometry, thin layer and column chromatography.

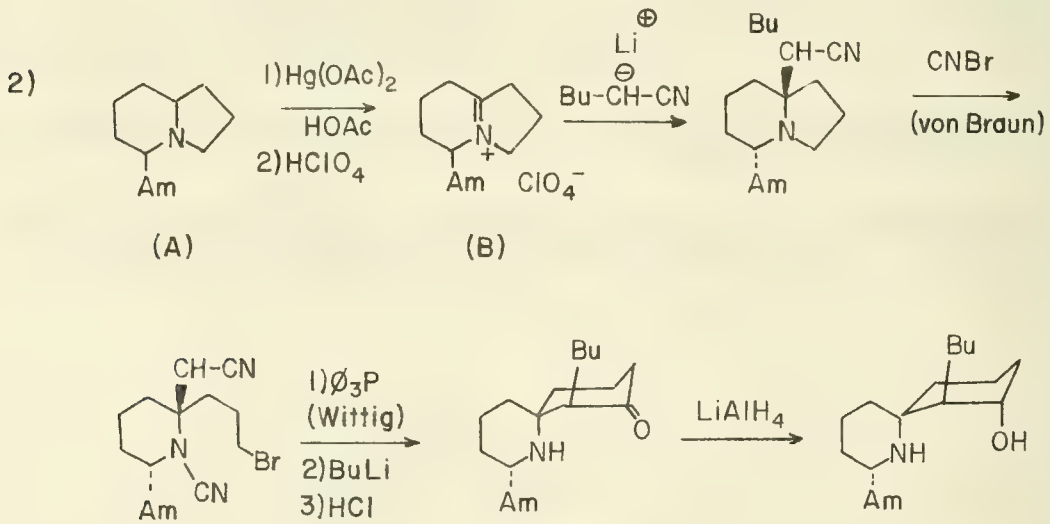
Major Findings: I. Synthetic approaches to Perhydrohistrionicotoxin (I)-a naturally occuring toxin of the frog *Dendrobates histrionicus*



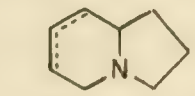
I



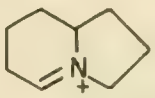
Scheme 1 was abandoned when several routes to derivatize the vinylogous amide nitrogen in (A) failed. Benzoylation, acetylation, trimethylsilylation and cyanoethylation (with CH_2 = CH-CN) were attempted.



(A) was synthesized in fair (30-40%) yield by a modification of a published procedure. Interestingly this synthesis does not produce the saturated indolizidine as claimed but rather gives exclusively unsaturated material (C). A separate step of catalytic reduction converted (C) to (A). Contrary to expectations



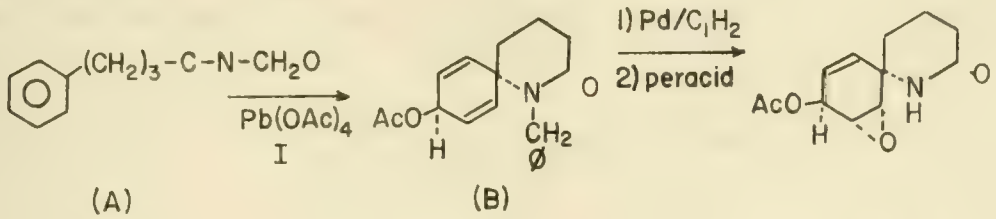
(C) Am

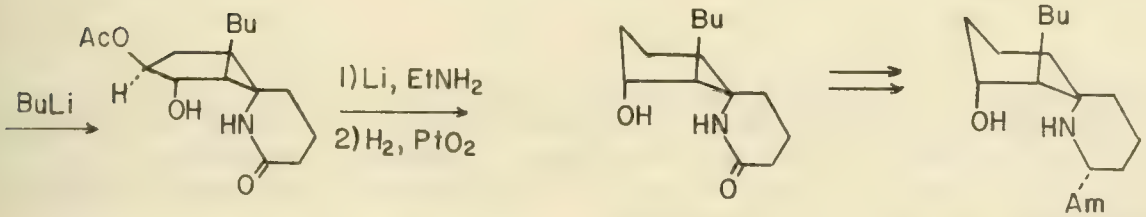


(D) Am ClO_4^-

from theory, mercuric acetatic oxidation of (A) gave mainly (D) accompanied by minor amounts of (B). A separation procedure was developed using LH-20 Sephadex chromatography; however the instability of (B) and its low yield caused this attractive route to be abandoned. Attempts to isomerize (B) to (D) using BF_3, anhydrous $HClO_4$ in CH_2Cl_2 or acidic silica or alumina were unsuccessful.

3)



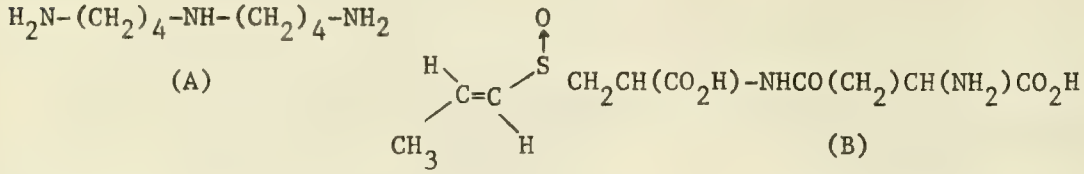


We are currently attempting the cyclization of (A) *via* the amido radical ($-CONCH_2O$), forming this in situ from the N-iodo amide. Should this reaction succeed and deliver the expected dienic spiroamine (B), the ensuing reactions will be attempted.

II. Natural Products from Sandal (*Santalum album* L).

In collaboration with biochemists at the Wellcome Research Institute, Tamil Nada, India, we have elucidated the structures of two materials, symmetrical homospermidine (A) and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine sulfoxide (B), from leaves of the Sandalwood tree. Each constitutes approximately 0.5% by weight of the dried leaves. The polyamine has not been found previously in nature while a dipeptide corresponding to *B* has been reported

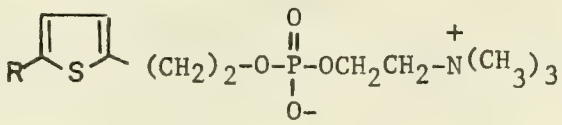
in onion. We were able to show by circular dichroism measurements that the Sandal and onion peptides were of opposite configuration at the sulfoxide group.



(A) (B)

III. The Nature of the Combining Site in a Homogeneous Myeloma Antibody.

In a collaboration with X-ray crystallographers (E. Padlan, D. Segal and D. Davies), a heavy atom-containing hapten was synthesized for use in locating the binding site of a crystalline myeloma (mouse plasmacytoma) antibody with phosphorylcholine binding activity isolated, purified and characterized by M. Potter and S. Rudikoff. A thiophene-ethanol ester of phosphorylcholine was prepared in a 3-step reaction (65% overall yield) and mercurated selectively and in high (94%) yield with mercuric acetate in methanol. A simple yet effective purification of B was achieved using LH-20 chromatography. The antibody bound two moles of B per mole, one of which was shown to be reversibly bound at the combining site - permitting for the first time the location and visualization of the antigen-binding site in an antibody.



A) R=H
B) R=Hg^{+} OAc^{-}

Significance to Biomedical Research and the Program of the Institute: Significant quantities of synthetic histrionicotoxin will be required to conduct pharmacological studies on its mode of action, to attempt by affinity-chromatography to isolate receptors and to study the effect of simple chemical modifications on the action of the toxin.

Proposed Course: Sym-homospermidine will be tested for tumor-inhibiting activity. The possible genesis of the sandal peptide from glutathione will be studied. A site-specific alkylating agent based upon a phosphorylchloline ester will be synthesized and studied with the mouse myeloma protein.

Honors and Awards: None

Publications: R. Kuttan, A. N. Radhakrishnan, T. Spande and B. Witkop, "Sym-Homospermidine, a Naturally Occurring Polyamine", *Biochemistry, 10,* 361 (1971).

R. Kuttan, N. G. Nair, A. N. Radhakrishnan, T. F. Spande, H. J. Yeh and B. Witkop, "The Isolation and characterization of -L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine sulfoxide from Sandal *(Santalum album* L). An

Interesting Occurrence of Sulfoxide Diastereoisomers in Nature."; submitted for publication.

E. A. Padlan, D. M. Segal, T. F. Spande, D. R. Davies, S. Rudikoff and M. Potter, "Structure at 4.5A Resolution of a Phosphorylch[illegible]-binding Fab", *Nature, New Biology, 245*, 165 (1973).

Serial No. NIAMDD-LC-30
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Service Function, Section on Microanalytical Services and Instrumentation

Previous Serial No.: NIAMDD, LC, #25

Principal Investigator: David F. Johnson

Other Investigators: P. Parisius, Byron Baer, A. Wong, W. R. Landis, H.Y.C. Yeh, N. Whittaker, B. Miller

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Basic research and service functions are performed by members of the Section. A major mission of the organization involves the instrumental and chemical analyses provided to scientists of the Laboratory of Chemistry, NIH, and to a limited extent to personnel of other government agencies. Approximately 30 of the more common elements and five functional groups are routinely determined on a quantitative basis, using ultramicro, micro, and semi-micro techniques as required. The materials analyzed include organic and inorganic research samples, commercial preparations and various biological specimens. Molecular weights are determined by vapor pressure osmometry in both aqueous and nonaqueous solvents. Instrumental analyses include: mass spectrometry, infrared, nuclear magnetic resonance, ultraviolet and flame photometry. Chemical analyses are done by most of the commonly used techniques including: gravimetric, colorimetric, gasometric, coulometric, potentiometric and volumetric. Assistance in the interpretation of spectra is rendered on request.

During the past year the total number of analyses performed in the microanalytical laboratory was 11,152, roughly a 16% decrease in the total from the

last reporting period. Review of the special type of analyses listed below reflects substantial increases in certain areas and decreases in others.

	1972-1973	1973-1974	% Change
Carbon	2517	2605	+3.5
Hydrogen	2517	2605	+3.5
Nitrogen	7000	4833	-31.0
Phosphorous	166	110	-33.7
Sulfur	78	67	-14.1
Halogens	293	483	+64.9
Metals	270	93	-65.6
Weight Loss	61	86	+41.0
Rotations	--	13	--
Miscellaneous	354	257	-27.4
Total	13,256	11,152	-15.9

It is apparent that the services are being utilized at a continuing high level. The distribution of service rendered is LC (41%) NIH, not including LC (57%), and outside agencies (2%).

The NMR service includes both the Varian A60 and HA-100. Over 500 spectra were run on the A60 by Mr. Benjamin Miller, who has recently been trained as a technician in this technique. Mr. Miller works under the guidance and supervision of Mr. Noel Whittaker and Mr. Miller continues to maintain the documentation and stocking of chemical stores.

Routine infrared spectral analysis has declined markedly over the previous year (120 to 63). This figure reflects the occasional need for specialized studies that cannot be performed on smaller Infracord IR. Mr. Whittaker continues to perform this service on request.

In excess of 1450 samples were submitted for mass spectral analysis. This includes analysis on the Hitachi Perkin-Elmer RMU 6-E Mass Spectrometer and the recently acquired Finnigan GC/MS computer system. Each analysis on the Hitachi involved several spectral determinations, depending on individual need, including: high resolution mass measurement, involving molecular-ion exact mass determination; isotope quantitative mass-ratio measurement; on-scale spectra recorded either/or the visicorder and the pen recorder; sample mixtures requiring fractional volatility-changing rate separations; and standard qualitative spectra. A collaborative research project was completed by Mr. Landis in cooperation with the Naval Medical Research Institute involving the identification and determination of Schistosoma Mansoni cercarial stimulation by crude egg lecithin and other lipids using the Hitachi instrument.

Mr. Noel Whittaker is the principal operator of the Finnigan GC/MS system. This instrument has been used heavily by investigators in LC on a variety of problems. The combined Gc capability, quadrapole rapidity, and computer storage and retrieval has been applied to such problems as: separation and identification of reaction products from reduction of benzopyrans; identifica-

tion of metabolites of bromoanisoles; and separation and identification of adrenal steroid mixtures to name a few. Use of this system is increasing daily as the potential of application becomes fully known.

The Section Chief continues to serve as Project Officer on Contract PH 43-65-32 between NIAMDD and the Medical Research Council of England. This contract is for the partial support of a program which provides reference steroid compounds to qualified research workers in the United States as well as to foreign investigators.

Publications: None.

Serial No. NIAMDD-LC-31
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: 1) Applications of NMR to the Structural Elucidation of Synthetic Drugs, Metabolites and Natural Products.
2) Nuclear Magnetic Resonance Studies of Reaction Rate, Molecular Motion and Molecular Interaction in Biochemical and Biological Systems.

Previous Serial No.: NIAMDD, LC, # 27, 29, 32, 33, 34, 38, 39

Principal Investigator: Herman J. C. Yeh

Other Investigators: R. Katz, D. F. Johnson, T. Oh-ishi, A. E. Jacobson, R. S. Wilson, E. L. May, A. M. Jeffery, D. M. Jerina, H. Ziffer, T. F. Spande, K. L. Kirk, L. A. Cohen, H. Yagi, B. Witkop.

Cooperating Units: R. M. DeMarinis, C. H. Foster, D. E. Piccolo and G. A. Berchtold of Massachusetts Institute of Technology; R. Kuttan, N. G. Nair and A. N. Radhakrishnan of Christian Medical College Hospital, Tamil Nadu, India.

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: 1) To examine and prove the stereochemical structure of biological important compounds such as drugs, metabolites and natural products. 2) Knowledge of the reaction rate, molecular motion as well as molecular interaction is of great importance in biochemical and biological research studies. The nmr spectroscopy has proven itself to be one of the most powerful tools to obtain this knowledge and has been used extensively in studies described below.

Methods Employed and Major Findings: Various nmr techniques have been

used for structural determinations and various other studies including the the following: 1) Stereochemical assignments of compounds, such as th reaction products resulting from action of nucleophiles upon arene oxides (known as causative agents in carcinogenesis), 2,9β-dimethyl-6,7-benzomorphan (having appreciable analgesic activity) and γ-L-glutamyl-S-(trans-1-propenyl)-L-cysteine sulfoxide (isolated from sandal leaves).

2) A fast equilibrium between oxide and oxepin tautomers is known to exist in a series of mono- and di- substituted benzene oxides. The existence of the valence tautomerism with comparable concentrations of the tautomers can be clearly shown by variable temperature nmr investigations, which can also be used to deduce the thermodynamic and kinetic parameters of the equilibrium. By employing nmr techniques, the preliminary result of this study indicates that the position of the equilibrium depends on solvent and on the position of substitution. Solvent with high dielectric constant or molecules with substitution at the β-position is found to shift the position of equilibrium toward the oxide tautomer.

3) The interaction between protonated amines (e.g. biogenic amines, amino acids) and organic phosphate containing substrates (e.g. Vitamin B_6 phosphates, adenosine triphosphate) has been studied by ^1H nmr. Although the results of these studies implied the existence of phosphate-amine interactions, informations obtained from ^1H nmr studies, however, offered little evidence about the phosphate-amine direct interaction. Using a $\{^{31}P\}$-^1H nmdr technique and pD titration, we are able to evaluate these interactions quantitatively, thus indicating the greater usefulness of the ^{31}P over the ^1H nmr method for this purpose, Study of the interaction between the phosphate group of 4-deoxypyridoxine-5 -phosphate (4-dPNP) and different protonated amines by ^{31}P nmr shows that basic amino acids and biogenic amines have significant interactions with the 4-dPNP. No interactions are observed for acidic or neutral α, β and γ-amino acids. Among the 19 biogenic amines studied, norepinephrine shows the strongest interaction with 4-dPN and mono-methyl phosphate.

4) Nmr chemical shifts of the ring protons of histidine has been widely used as a probe for the studies of local structure of peptides and proteins. It is known that ^{19}F nucleus is more sensitive than ^1H nucleus for probing the local charge effects. We have used ^{19}F and ^1H nmr to investigate the effect of local charge on ^{19}F and ^1H chemical shifts in 2-fluoro and 4-fluoro histidines, which, from some early studies, have indicated their biological significance in analogy to histidine. A large shift in ^{19}F over ^1H nmr signals is observed in both compounds as the side chain groups of these molecules being titrated, illustrative of the high sensitivity of ^{19}F signals to distant field effects. In complex systems, like proteins, the use of ^{19}F as an nmr reporter also offer an advantage over that of ^1H, since overlap or concealment by neighboring signals can be eliminated. Present study suggests that the use of ^{19}F nmr for the study of protein systems is encouraging.

Significance to Biomedical Research and the Program of the Institutes:
Nuclear magnetic resonance proves very useful for the structural elucidation

of compounds, (such as drugs, metabolites and natural products) as well as for the studies of various biochemical and biological systems. Results of these studies provide useful information for the future biomedical research

Proposed Course: Various nmr techniques will continuously be used 1) structural determination on biologically important compounds; 2) the conformational study of macromolecules, and 3) the study of subtrate-enzyme interaction.

Publications: Katz, R., Yeh, H.J.C., and Johnson, D.F.: A quantitative study of organic phosphate-amine interaction by ^{31}P nuclear magnetic resonance. Biochem. Biophys. Res. Comms., in press 1974.

Oh-ishi, T., Jacobson, A. E., Wilson, R. S., Yeh, H. J.C., and May, E. L.: Synthesis of 2,9β-dimethyl-6,7-benzomorphan. J. Org. Chem., in press 1974.

Jeffery, A. M., Yeh, H. J.C., and Jerina, D. M.: Synthesis of cis-1,2-dihydroxy-1,2-dihydronaphthalene and cis-1,4-dihydroxy-1,4-dihydronaphthalene. J. Org. Chem., in press 1974.

Jeffery, A. M., Yeh, H. J.C., Jerina, D. M., DeMarinis, R. M., Foster, C. H., Piccolo, D. E., and Berchtold, G. A.: Stereochemical course in reactions between nucleophiles and arene oxides. J. Amer. Chem. Soc., in press 1974.

Serial No. NIAMDD-LC-32
1. Chemistry
2. Microanalytical Services
& Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Steroid Transformations by Tetrahymena Pyriformis

Previous Serial No.: NIAMDD, LC, #28

Principal Investigator: Nancy S. Lamontagne

Other Investigator: David F. Johnson

Cooperating Unit: Chester E. Holmlun, Department of Chemistry,
University of Maryland, College Park, Maryland

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: The above mentioned cooperating unit has been interested in the growth inhibitory effects of steroids on the protozan Tetrahymena pyriformis. Previous studies demonstrated that a variety of steroids of mammalian origin, as well as steroid derivatives such as dialkylaminoethoxy steroids inhibit the growth of T. pyriformis, and that this growth inhibition is readily reversed by the addition of cholesterol to the medium. The mode of action of these growth inhibitory steroids is not definitely known, but inhibition of NADH oxidation and RNA synthesis, and interference with respiration and formation of lipids have been demonstrated. Of special importance in T. Pyriformis metabolism is the lipid tetrahymenol, a pentacyclic triterpenoid alcohol produced in the organism by a direct, non-oxidative, proton initiated cyclization of squalene. T. pyriformis grown without added steroids produces tetrahymenol as its major unsaponifiable product. When steroids such as dehydroepiandrosterone are added to the culture an accumulation of squalene occurs with a corresponding decrease in growth rate of the organism. It has been suggested that cholesterol overcomes this inhibitory effect by performing the role (as yet unknown) of tetrahymenol in the metabolism of T. pyriformis. Since the focus of attention has always been

on the action of steroids on tetrahymena, it was decided that the reverse study, i.e., the effect of tetrahymena on steroids would give additional information on the role of steroid metabolism in Tetrahymena pyriformis.

Methods Employed and Major Findings: Radioactive steroid precursers are added to T. pyriformis cultures in the exponential phase of growth. The inoculated cultures are incubated for 24 or 48 hours. Centrifugation is used to separate the cell pellet and media, which are then extracted, assayed for radioactivity, and chromatographed by column and thin-layer techniques to separate and identify the radioactive metabolic products.

All of the substrates incubated (androstenedione, progesterone, 17α-OH-progesterone, testosterone, corticosterone and pregnenolone) have yielded metabolites, as indicated by separate peaks on column and thin-layer chromatography. Tentative identifications indicate hydroxylation, reduction of a carbonyl group to a hydroxyl, and isomerization as possible transformations.

Proposed Course: Thin layer analyses will be completed to yield tentative identification of as many metabolites as possible. Derivative formation, gas chromatography, and mass spectra analyses will then be used for verifications.

Publications: None

Serial No. NIAMDD-LC- 33
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
October 15, 1973 through June 30, 1974

Project Title: Metabolism of C^{14}-Labelled Steroids by Adrenals from Rats with a Mammotropic Pituitary Tumor

Previous Serial No.: NIAMDD, LC, #30

Principal Investigator: B. A. Weissman

Other Investigator: David F. Johnson

Cooperating Unit: Robert W. Bates (NIAMDD-LNE)

Man Years:
Total: 0.7
Professional: 0.7
Other: 0

Project Description:

Objective: Qualitative, quantitative and semi-kinetic study of steroid biogenesis in rat adrenals subjected to long-term stress by ACTH, growth hormone, and prolactin.

Methods Employed: Radioactive C^{14}-progesterone and C^{14}-pregnenolone were incubated with adrenals from rats subjected to long-term stress of tropic hormones of the anterior pituitary. Substrates were incubated for 1 and 2 hours. Extracts (from the media as well as tissues) were chromatographed on short silicic acid columns. The radioactivity zones from the columns are then further characterized by thin-layer while another portion is converted to silyl derivatives for GC-MS identification.

Major Findings: The preliminary data shows that as demonstrated earlier by studies in this laboratory, the conversion of either progesterone or pregnenolone by the adrenal tissue from tumor animals is markedly reduced. Qualitative and quantitative studies are being carried out now to establish the pathways of steroidogenesis in these adrenal tissues.

Significance to Biomedical Research and the Program of the Institute: The tumor-bearing rats show lowered blood cholesterol and splanchnomegaly of the liver, kidney, and heart. The fact that splanchnomegaly does not occur in adrenalectomized rats with MtT, and administration of adrenal steroids to adrenalectomized MtT rats did not exhibit splanchnomegaly, suggested that some unknown factor of adrenal origin was the possible causative agent. Present work is designed to investigate the origin of this factor.

Proposed Course: Continuation of studies to elucidate the biosynthetic pathway exhibited by adrenal tissue from MtT rats, using pregnenolone as substrate, and identification of metabolites produced.

Publications: None

Serial No. NIAMDD-LC-34
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The study of the Constituents of Solanum Xanthocarpum

Previous Serial No.: NIAMDD, LC, #31

Principal Investigator: Genjiro Kusano and Robert Katz

Other Investigators: Yoshio Sato and David F. Johnson

Cooperating Unit: Malcolm J. Thompson and William E. Robbins, Insect Physiology Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland

Man Years:
Total: 0.4
Professional: 0.4
Other: 0

Project Description:

This project is terminated.

Serial No. NIAMDD-LC-35
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Titles: 1) Chemical structure - biological activity correlations in the enzymatic reactions of catechol-O-methyltransferase (COMT) with different substrates.
2) Molecular Orbital studies of biologically important amines.
3) Molecular Orbital studies of important biochemical intermediates (chorismic acid and derivatives).

Previous Serial No.: NIAMDD, LC, #26

Principal Investigator: Robert Katz

Other Investigators: A. E. Jacobson, S. R. Heller, R. J. Feldman

Cooperating Units: DCRT

Man Years:
Total: 0.4
Professional: 0.4
Other: 0

Project Description:

These projects have been terminated.

Serial No. NIAMDD-LC-36
1. Chemistry
2. Microanalytical Services & Instrumentation
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The development of chemical methods and the synthesis and study of chemical compounds of use in the investigation of biological and medical problems.

Previous Serial No.: NIAMDD, LC, #35

Principal Investigator: Calvin M. Foltz

Other Investigators: None

Cooperating Units: None

Man Years:
Total: 1
Professional: 1
Other: 0

Project Description:

Objective: To develop organic chemical and photochemical methods, to synthesize chemical compounds, and to study the properties of compounds of potential utility in the investigation of biological and medical problems. To study the mechanism of the photocyclodehydrohalogenation produced by UV irradiation of N-chloroacetyltryptamine and related compounds and to study the scope of its applicability to other heterocyclic and non-heterocyclic systems. To study the mechanism, scope, and applicability of the rearrangement which was discovered on irradiation of 3-(3'-chloro-2'-oxopropyl)indolin-2-one with a mercury lamp. To study the photo-Fries rearrangement of esters, particularly of halo-acids, with a view to developing methods for modifying and synthesizing compounds of potential interest. To develop methods for the synthesis of organoselenium compounds and to synthesize and study the chemical and physical properties of such compounds of particular biological interest.

Methods Employed: Standard organic chemical techniques; irradiation of solutions of organic compounds with UV radiation from mercury lamps; column, gas, and thin layer chromatography; and visible, UV, IR, NMR, and mass spectroscopy.

Major Findings: Photolysis of N-chloroacetyl derivatives of tryptamine (I), 3-(3'-aminopropyl)indole (II), and 3-(4'-aminobutyl)indole (III) with a high pressure mercury lamp results in the formation of high-melting dehydrohalogenation products in yields of 50%, 30%, and 20%, respectively. The product of the photolysis of the N-chloroacetyl derivative of I is the tricyclic lactam, 6-oxo-3,4,6,7-tetrahydro-H,5H-azocin[4,5,6-c,d]indole, which results from cyclization in the 4-position of the indole moiety. NMR spectroscopy of the amine produced by the diborane reduction of the lactam provided conclusive evidence for the position of the cyclization. The amide and the amine are also of interest because of their potential biological activities.

Analytically pure photodehydrohalogenation products have been obtained from the photolyses of N-chloroacetyl derivatives of II and III. It is expected that spectroscopic methods applied to these products or the amines derived from them will permit structural assignments. A second crystalline product has been obtained, but not yet characterized, from column chromatography of preparative photolyses of the N-chloroacetyl derivative of II. Diborane reductions of the principal product have yielded an amine in poor yield, which has been isolated with considerable difficulty thus far and which has not yet been characterized. As a part of the study of this photodehydrohalogenation reaction N-chloroacetyl-2-(α-naphthyl)ethylamine was preapred as a prototype of polycyclic aromatic systems and photolyzed with a mercury lamp. A tricyclic lactam, 2-oxo-2,3,4,5-tetrahydro-1H-naphth)1,8-d,e)azocine, in 47% yield. N-Chloroacetyl derivatives of compounds such as aniline, benzylamine, β-phenylethylamine, 1-aminomethylnaphthalene, α- and β-naphthylamines also have been prepared and phototitrations carried out. These studies have not yet been completed. The utility of the automatic phototitration technique was discovered in the study of photodehydrohalogenations and has been applied to several series of compounds. The kinetic data obtained in this way provide useful information on the course and mechanism of such reactions. Further refinements of the technique to extend its usefulness are to be made. Several years ago in the course of studies of intermolecular photodehydrodehydrogenation reactions, anomalousphototitration results were obtained with phenyl choroacetate as one of the reactants. A photo-induced Fried rearrangement followed by cyclodehydrodehalogenation was apparently taking place. This reaction may afford a mild specific way for synthesizing or modifying certain compounds of biological interest and studies of the reaction are in progress.

In 1957 the discovery of the fact that dietary agent, Factor 3, which prevented dietary liver necrosis in the rat, supplied trace amounts of seleenium was reported (K. Schwarz and C. M. Foltz, J. Am. Chem. Soc., 79, 3292 (1957)]; at that time it was inferred that selenium might act in intermediary metabolism by participating in oxidation-reduction reactions. This report stimulated a large and growing body of work on the nutritional role of selenium in livestock, laboratory animals, and poultry; on the biological role of selenium and its relationship to that of Vitamin E; and on the chemistry of organoselenium compounds. Recently it has been reported that two enzymes involved in oxidation-reduction reactions contain selenium; namely, glutathione peroxidase and glycine reductase, and a selenoprotein which may participate in

such reactions has been isolated from lamb muscle (reviewed by T. Stadtman, Science, 183, 915 (1974)]. These facts are some of the considerations which have sustained a continuing interest in the biological roles and the chemistry of selenium compounds. As circumstances permit, work on the chemistry of selenium compounds is contemplated.

Significance to Biomedical Research and the Program of the Institute
New compounds produced in this work are of biological and medical interest. The organic and photochemical reactions being developed can be applied to the synthesis of other compounds of biological and medical interest, to the modification of compounds of biological and medical interest, and to the development of methods for use in the study of biological and medical problems.

Proposed Course: To continue to study the mechanisms and scope of applicability of the reactions described in this report. To apply the methods developed to the synthesis of compounds of particular biological or medical interest. To develop and study other chemical reactions and techniques with potential utility for the synthesis, modification, characterization, and study of molecules of biological and medical interest. To develop chemical methods which can be applied in the study of biological and medical problems. To synthesize compounds of potential biological and medical interest.

Publications: None

Serial No. NIAMDD-LC-37
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Role of Cyclic Adenosine Monophosphate in the Central Nervous System

Previous Serial Number: NIAMDD-LC-47

Principal Investigator: John Daly

Other Investigators: P. Skolnick, H. D. Mah, K. Dismukes

Cooperating Units: F. Bloom, G. Siggins, and B. Hoffer, St. Elizabeths Hospital, NIMH
E. Gruenstein, M.I.T., Cambridge, Massachusetts
V. Manganiello, IR:MD-NHLI

Man Years

Total: 3.9
Professional: 3.6
Other: 0.3

Project Description:

Objectives: To determine the factors that govern the formation and metabolism of cyclic AMP in the central nervous system. To investigate the possible role of cyclic AMP in regulating biochemical reactions and membrane properties in brain tissue.

Methods Employed: Isotopically labeled compounds have been used to study the formation and breakdown of adenine nucleotides in brain slices slices and with partially purified enzymes.

Major Findings: Pretreatment of rats with agents which disrupt noradrenergic input in the central nervous system results in a hypersensitivity of cyclic AMP-generating systems to norepinephrine. Thus, a 1.5-3 fold increase in the accumulation of cyclic AMP elicited by norepinephrine pertains in cerebral cortical slices from animals in which i) noradrenergic terminals have been destroyed by 6-hydroxydopamine ii) central levels of norepinephrine have been reduced by reserpine and iii) norepinephrine synthesis has been inhibited by α-methyl-p-tyrosine. Both α- and β-

adrenergic receptor-mediated mechanisms appear to be enhanced by such treatments.

Responses of norepinephrine-sensitive cyclic AMP generating systems in combined midbrain-striatal slices of four rat strains correlated positively with spontaneous behavioral activity and negatively with levels of midbrain and striatal tyrosine hydroxylase. Responses of cerebral cortical norepinephrine-sensitive cyclic AMP systems correlated negatively with spontaneous behavioral activity and positively with midbrain and striatal tyrosine hydroxylase. Such correlations were not found with responses of the cyclic AMP-generating systems to isoproterenol, adenosine, veratridine, or of an adenosine and norepinephrine combination. The results suggest that in animals in which the genetically controlled levels of neurotransmitter synthesis and release are low, there has been a compensatory increase in receptor-mediated response systems. The results also provide further evidence for the importance of noradrenergic systems to levels of spontaneous behavioral activity.

Clonidine, a compound proposed to regulate blood pressure, via interaction at central presynaptic α-adrenergic receptors was found to be an α-antagonist rather than an α-agonist with respect to generation of cyclic AMP in rat cerebral cortical slices.

A variety of studies have established that depolarization of neuronal structures results in a lowering of levels of ATP in brain slices along with an increase in 5'-AMP and adenosine. The adenosine then appears to stimulate cyclic AMP formation in other morphological entities. Such conclusions are based in part on differential effects of depolarizing agents, metabolic inhibitors and psychotropic agents on levels of ATP, adenosine and cyclic AMP. In addition, studies of brain slices with adenosine deaminase clearly demonstrate that adenosine must be released during depolarization of brain cells into extracellular space before stimulating cyclic AMP formation. Adenosine stimulates intracellular formation of cyclic AMP by interaction with an extracellular receptor as clearly shown by potentiation of the stimulatory effects of low concentrations of adenosine by agents which nearly completely block its uptake into the tissue.

A variety of adenine and adenosine analogs have been studied with brain slices as inhibitors of adenine and/or adenosine incorporation and in certain instances as possible precursors of cyclic AMP analogs in the intact cells of these preparations. The fluorescent ethanoadenine and ethanoadenosine analogs were not effectively incorporated into slices. Radioactive N^6-benzyladenosine was readily incorporated into slices, but only the 5'-monophosphate was detected as a product. Radioactive 2'-deoxyadenosine was incorporated into intracellular nucleotides which served as precursors of 3',5'-cyclic phosphates. However, virtually all incorporation of this nucleoside was found to have been preceded by enzymatic hydro-

lysis to radioactive adenine so that radioactive cyclic AMP rather than radioactive 2-deoxy-cyclic AMP was the major cyclic nucleotide formed on stimulation of prelabeled slices. Radioactive 2-chloroadenine was prepared and is under investigation.

Significance to Biomedical Research and the Program of the Institu
The key role of adenyl cyclase and cyclic AMP in regulating cellular activity in response to external stimuli in many biological systems make elucidation of their role in brain of fundamental importance to an understanding of function in this organ.

Proposed Course of Project: The morphological location of the labeled pools of adenine nucleotides will be determined. The role of phosphoribosyl transferase and adenosine kinase in maintaining this pool will be explored. Secondary effects due to elevated cyclic AMP levels in brain tissue will be investigated. The in vivo effects of drugs and the effects of lesions on the responsiveness of the cyclic AMP system will be further explored. Attempts to label the precursor pool with fluorescent and radioactive adenine analogs will be continued. The nature of the adenosine receptor will be studied further using adenosine analogs modified for possible affinity labeling. Other agents with selective effects on adrenergic, serotonergic and histaminergic systems will be sought. The role of calcium and other ions in the function of cyclic AMP-generating systems will be investigated.

Honors and Awards: None

Publications:

Huang, M., Gruenstein, E., and Daly, J. W.: Depolarization-evoked accumulation of cyclic AMP in brain slices: Inhibition by exogenous adenosine deaminase. Biochim. Biophys. Acta 329: 147-151, 1973.

Schultz, J. and Daly, J. W.: Accumulation of cyclic adenosine 3',5'-monophosphate in cerebral cortical slices from rat and mouse: Stimulatory effect of α- and β-adrenergic agents and adenosine. J. Neurochem. 21: 1319-1326, 1973.

Huang, M., Ho, A. K. S., and Daly, J. W.: Accumulation of adenosine cyclic 3',5'-monophosphate in rat cerebral cortical slices. Stimulatory effect of alpha- and beta-adrenergic agents after treatment with 6-hydroxydopamine, 2,3,5-trihydroxyphenethylamine, and dihydroxytryptamines. Mol. Pharmacol. 9: 711-717, 1973.

Huang, M. and Daly, J. W.: Adenosine-elicited accumulation of cyclic AMP in brain slices: Potentiation by agents which inhibit the uptake of adenosine. Life Sciences 14: 489-503, 1974.

Skolnick, P. and Daly, J. W.: Norepinephrine-sensitive adenylate cyclases in rat brain: Relation to behavior and tyrosine hydroxylase. Science 184: 175-177, 1974.

Daly, J. W.: Regulation of cyclic AMP levels in brain. In Snyder, S. H. and Usdin, E. (Eds.). Frontiers in Catecholamine Research. New York, Pergamon Press, 1974, pp. 301-306.

Daly, J.: The role of cyclic nucleotides in the nervous system. In Iversen, L. L., Iversen, S. E. and Snyder, S. H. (Eds.). Handbook of Psychopharmacology. New York, Plenum Publishing Corp., 1974, in press.

Huang, M. and Daly, J. W.: Enhanced accumulations of cyclic AMP in brain slices elicited by norepinephrine after intraventricular pretreatment of rats with 6-hydroxydopamine. In Fuxe, K., Olsen, J., and Zotterman, A. (Eds.). Wenner-Gren Symposium XXII "Dynamics of Degeneration and Growth in Neurons. New York, Pergamon Press, 1974, in press.

Skolnick, P. and Daly, J. W.: The accumulation of adenosine 3',5'-monophosphate in cerebral cortical slices of the quaking mouse, a neurologic mutant. Brain Research. In press.

Serial No. NIAMDD-LC-38
1. Chemistry
2. Oxidation Mechanisms
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Mechanism of Enzymatic Oxidation and the Role of Reactive Intermediates in Drug Metabolism, Hepatotoxicity and Carcinogenesis

Previous Serial Number: NIAMDD-LC-48

Principal Investigator: Donald M. Jerina

Other Investigators: J. Daly, H. Yagi, J. Tomaszewski, H. Selander, H. Yeh

Cooperating Units: D. Boyd, Univ. Belfast, Belfast, Ireland
H. Ziffer, NIAMDD:LPB
O. Chapman, Iowa State Univ., Ames, Iowa
A. Y. H. Lu, A. Conney, A. Wood, and W. Levin, Hoffmann-La Roche, Nutley, New Jersey
D. W. Nebert, NICHHD:LBS
T. C. Bruice, Univ. of California, Santa Barbara, Calif.
D. T. Gibson, Univ. of Texas, Austin, Texas
G. Berchtold, M.I. T., Cambridge, Massachusetts
F. McLafferty, Cornell Univ., Ithaca, New York
P. Dansette, Guest Worker, NIAMDD:LC
A. Jeffrey, NIAMDD:LBP
S. Milstien, NIMH:LNC
V. Makedonska, Guest Worker, NIAMDD:LC
G. Holder, Fogarty Scholar, NIAMDD:LC
S. Chu, Roche Fellow, NIAMDD:LC
B. Weinstein, Columbia University, New York, N. Y.

Man Years

Total: 4.3
Professional: 4.0
Other: 0.3

Project Description:

Objectives: To study the basic mechanism of oxygen activation and enzymatic incorporation into organic substrates. To explore enzymatic and non-enzymatic reactions of labile intermediates such as epoxides and

cyclic peroxides. To devise methods for the synthesis and study of the chemical reactions of arene oxides. To investigate the significance of labile oxygenated intermediates to the cytotoxicity and carcinogenicity of xenobiotic substances.

Methods Employed: Isotopically labeled substrates, water and oxygen gas, have been to study the oxidation of organic compounds with a variety of enzymes and the subsequent metabolism of labile intermediates. Nuclear magnetic resonance spectroscopy, mass spectrometry, x-ray crystalography, and other physico-chemical techniques have been employed frequently in these studies. In addition to the usual techniques employed for the separation of metabolites, high pressure liquid chromatography is being pioneered in this area.

Major Findings: A major portion of the efforts of this section have been directed toward elucidation of fundamental mechanisms of drug metabolism in mammals. To this end, a substantial effort is being directed at the hydrocarbon benzo[a]pyrene (BP) for two basic reasons: BP is the most prevalent environmental carcinogen to man, and the confusion in the 70 existing papers on the metabolism of this hydrocarbon prove the need for a multidisciplinary approach to the problem. The first phase of this approach has been purely chemical in that 12 phenols, 6 arene oxides, 6 dihydrodiols, and a multitude of quinones were required as reference standards for the enzyme studies. About three fourths of these materials are now in hand. The unusual chemistry of the polycyclic hydrocarbons has prompted development of new synthetic methods for production of these compounds. In particular, halohydrin esters have been exploited as latent precursors to both K-region and non-K-region arene oxides. Specialized methods of phenol synthesis particularly applicable to polycyclic hydrocarbons have been devised.

In order to explore metabolism of BP, high pressure liquid chromatography has been exploited to separate various classes of metabolites. Preliminary results have been extremely satisfying in that they show prior methods of analysis take into account only 10-30% of the actual metabolites. Studies with liver microsomes, solubilized and purified drug metabolizing systems (P-450 and P-448), and bacteria show marked differences. These differences will be pursued in light of species and organ susceptibility to the action of this carcinogen. A variety of previously undetected metabolites have been isolated.

A broad screening program for carcinogenic and transforming properties of the suspected carcinogenic metabolites has been undertaken. Testing includes topical application, subcutaneous injection, and transformation of cells in culture. Statistically significant data should be available within 12 months.

Two classes of enzyme systems, epoxide hydrase and glutathione S-epoxide transferase, are known to detoxify metabolically formed arene oxides which are the suspected bioactivated carcinogens. Phenobarbital was found to induce benzy[a]pyrene hydroxylase activity in seven strains of mice while inducing epoxide hydrase in only four of these strains. 3-Methylcholanthrene induced the hydroxylase in four strains but did not induce hydrase. Studies on the mechanism of action of epoxide hydrase have established that these enzymes function by nucleophilic addition rather than acid catalysis as previously thought. Studies with the transferase have established that arene oxides from strained carcinogens are more susceptible to attack and detoxification. Further examination of the chemistry of arene oxides has established marked differences between the kinetics of isomerization and mode of nucleophilic addition of K-region and non-K-region oxides.

Interaction of arene oxides with nucleic acid has shown that non-K-region oxides are highly reactive toward DNA in contrast to results of earlier studies. An attempt to correlate reactivity with carcinogenicity is in progress.

Product ratios in metabolism of chlorobenzene and primary isotope effects in the metabolism of a variety of aromatic hydrocarbons have established that an unsuspected mode of direct hydroxylation occurs in mammalian liver. Hopefully, delineation of the factors involved, will allow an exploitation of this route of drug metabolism which circumvents formation of cytotoxic arene oxides.

Significance to Biomedical Research and the Program of the Institute: Enzymatic oxidation reactions are extremely important in many biosynthetic pathways and in the metabolism, detoxification and action of most drugs.

Proposed Course of Project: The mechanism(s) of enzymatic hydroxylation and epoxidation will be further investigated. Arene-oxides of other aromatic substrates will be synthesized and attempts will be made, using tracer techniques, to demonstrate their intermediacy in aryl oxidation. The mechanism of action of epoxide hydrase and glutathione transferase and their role in drug detoxification will be further studied. Assays for the hydration of polycyclic arene oxides will be developed and the relevant enzymes will be investigated. Attempts to relate the effect of labile arene oxides on cells grown in culture and in whole animals to the cytotoxicity and carcinogenicity of the parent hydrocarbons will be made. The effect of various parameters on the stereochemical opening of epoxides with the microsomal epoxide hydrases will be explored. Studies on model systems including photolysis of N-oxides will be continued.

Honors and Awards:

The accomplishments of the members of this section have been recognized in that Dr. Jerina is presently a Visiting Professor at SUNY Buffalo. In addition, the section has been awarded funds from Hoffmann-La Roche for the support of a postdoctoral fellow.

Publications:

Dansette, P. and Jerina, D. M.: A facile synthesis of arene oxides at the K-region of polycyclic hydrocarbons. J. Am. Chem. Soc. 96: 1224-1225, 1974.

Jerina, D. M. and Daly, J. W.: Mechanisms for the oxidative metabolism of the aromatic nucleus. In Ing, T. E. and Mason, H. S. (Eds.). Oxidases and Related Redox Systems. Proceedings of the Second International Symposium. University Park Press, Baltimore, 1973, pp. 143-166.

Jerina, D. M., Yagi, H. and Daly, J. W.: Arene oxides-oxepins. Heterocycles 1: 267-326, 1973.

Oesch, F., Morris, N., Daly, J. W., Gielen, J. E., and Nebert, D. W.: Genetic expression of the induction of epoxide hydrase and aryl hydrocarbon hydroxylase activities in the mouse by 3-methylcholanthrene and phenobarbital. Mol. Pharmacol. 9: 692-696, 1973.

Ziffer, H., Jerina, D. M., Gibson, D. T., and Kobal, V. M.: Absolute stereochemistry of the (+)-cis-1,2-dihydroxy-3-methylcyclohexa-3,5-diene produced from toluene by pseudomonas putida. J. Am. Chem. Soc. 95: 4048-4049, 1973.

Jerina, D. M. and Daly, J. W.: Formation, toxicity and carcinogenicity of arene oxides: A new aspect of drug metabolism. Science. In press.

Jerina, D. M.: Biological formation and disposition of arene oxides. Lloydia: In press.

Jeffrey, A. M., Yeh, H. J. C., and Jerina, D. M.: Synthesis of cis-1,2-dihydroxy-1,2-dihydronaphthalene and cis-1,4-dihydroxy-1,4-dihydronaphthalene. J. Org. Chem.: In press.

Selander, H. G., Jerina, D. M., and Daly, J. W.: Metabolism of acetanilide with hepatic microsomes and reconstituted cytochrome monoxygenase systems. Arch. Biochem. Biophys.: In press.

Serial No. NIAMDD-LC- 39
1. Chemistry
2. Pharmacodynamics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Pharmacodynamic Amines and Enzymes Involved in Their Metabolism

Previous Serial Number: NIAMDD-LC-49

Principal Investigator: Cyrus R. Creveling

Other Investigators: J. Daly and A. Rotman

Cooperating Units: K. Fuxe, Karolinska Institute, Stockholm, Sweden
R. Borchardt, University of Kansas, Lawrence, Kansas
B. Highman, Natl. Center Toxicological Res., Jefferson, Arkansas
B. Hartman, Washington University, St. Louis, Mo.
R. Katz, Andrulis Res. Corp., Washington, D. C.
G. Breese, University of North Carolina, Chapel Hill, N. C.
C. Sachs, Karolinska Institute, Stockholm, Sweden
I. Carter, NIAMDD-LBP
L. Tice, NIAMDD-LEP

Man Years

Total: 4.1
Professional: 1.8
Other: 2.3

Project Description:

Objectives: To study the basic chemistry and biochemistry of the catecholamines, phenolic amines, indolealkylamines, imidazolealkylamines and their precursors and transformation products. To develop new techniques for the investigation of transport metabolism and binding phenomena as related to these amines and their interactions with various drugs. To isolate and purify enzymes operative in the biosynthesis and metabolism of biogenic amines. To determine the physical properties of these enzymes and the effect of pH, temperature, substrate specificity, reversible and irreversible inhibitors and the ionic composition of the incubation media. To develop assays and to apply these enzymatic assays to determine specific

biogenic amines and to study the distribution and variation of enzyme levels. To develop new pharmacologically active agents for the study of the biological parameters that affect biogenic amines.

Methods Employed: Drugs and substrates labeled with tritium, deuterium or ^{14}C have been used to study the metabolic fate of endogenous amines and amino acids and the underlying mechanisms of uptake, release and metabolism of these compounds. Standard methods of enzyme purification have been used including: differential centrifugation, density gradient centrifugation, gel filtration, electrophoresis, standard column chromatography, immunodiffusion, immunoelectrophoresis and affinity labeled column chromatography.

Major Findings: More efficient procedures for the isolation of catechol-O-methyltransferase (COMT) have been developed. Antibodies to this enzyme have been prepared and used: to develop a radioimmunoassay for COMT; to study the species and organ distribution of COMT; to study the subcellular distribution and localization of COMT and with immunofluorescent techniques to study its distribution in brain and other tissues. A survey of rat brain with fluorescent anti-COMT revealed the presence of a fine nerve fiber network with clearly outlined dendritic terminals nonuniformly distributed in the areas examined. In addition several CNS structures were highly fluorescent, in particular the cysterna magna. The subcellular distribution and properties of membrane-bound COMT from liver microsomes have been investigated.

Further studies on the structure/activity correlations on the inhibition of uptake, release, and long term effects on the uptake mechanisms by phenethylamines, phenethanolamines and indoleamines in heart have been continued. It has been shown that 2-hydroxyphenethanolamines are virtually inactive as inhibitors of the norepinephrine uptake mechanism as compared to the highly effective 2-hydroxyphenethylamines. Consideration of the probable preferred conformation of these compounds derived from molecular orbital calculations suggest that the aromatic 2-hydroxy group is hydrogen bonded to the side chain amino-group. Such a gauche conformer appears to interact strongly with the uptake receptor site except in the presence of a β-hydroxy moiety where the interaction is blocked. The results of this study suggest that both the extended and the gauche conformers of dopamine can interact with the uptake site of noradrenergic nerve terminals in cardiac tissue, but only the extended conformer of norepinephrine has a high affinity. The results rationalize the lack of degenerative effects after administration of 6-hydroxynorepinephrine as contrasted to the marked degenerative effects elicited by 6-hydroxydopamine.

The neurodegenerative effect of 5,7-dihydroxy tryptamine has been confirmed by direct morphological examination by electron microscopy of sympathetic nerve in mouse atria and thyroid. The morphological appearance and time course of the degenerative is similar to that induced by 6-hydroxy-

dopamine. The prevention of the long term biochemical and morphological effects of 5,7-dihydroxytryptamine after prior inhibition of monoamine oxidase suggests that the proximate cytotoxic agent is formed by oxidation of the parent compound. 5,6-Dihydroxytryptamine after large dosages causes subepicardial lesions, while ischemic subendocardial lesions are elicited by 6,7-dihydroxytryptamine. 5,7-Dihydroxytryptamine did not elicit such sequelae.

The binding of radioactive 6-hydroxydopamine, dopamine, 5,7-dihydroxytryptamine, norepinephrine, tryptamine, serotonin and various other amines to bovine serum albumin has been studied. Only in the case of 6-hydroxydopamine did extensive covalent binding occur. The reaction of 6-hydroxydopamine with albumin result in extensive crosslinking to form a series of albumin polymers ranging from the monomer, dimer, trimer to polymers with molecular weights in excess of a million. This crosslinking reaction occurs thru the sulfhydryl groups of albumin and is absent with albumin where all the available sulfhydryl groups were blocked with N-ethylmaleimide. Radioactive 6-hydroxydopamine is taken up and bound covalently to at least three proteins in mouse and rat atrial preparations in vitro.

A simple, inexpensive procedure for the labeling of a variety of phenethylamines has been developed consisting of the oxidation of a precursor nitro alcohol to a ketone followed by reduction with sodium borotritide.

2,4-Dihydroxy-5-methoxyphenethylamine was synthesized to investigate a recent report that this compound elicits a long lasting depletion of dopamine in the central nervous system. No affinity for the peripheral norepinephrine-uptake system could be demonstrated.

The distribution, metabolism and incorporation into protein of ^{3}H-2-fluorohistidine in the mouse has been examined. 2-Fluorohistidine can substitute for histidine and gives rise to proteins containing 2-fluorohistidine in vivo.

Significance to Biomedical Research and the Program of the Institute: The biosynthesis and metabolism of biogenic amines are of fundamental importance to the fields of neurochemistry, pharmacology and have a direct bearing on the understanding of the functioning of the cardiovascular system.

Proposed Course of Project: The binding and release of norepinephrine as influenced by drugs will be explored further, as will interactions of certain amines and drugs at storage and receptor sites. Studies on active site labeling and the primary structure of catechol-O-methyltransferase will be continued. Studies on inhibitors of tyrosine hydroxylase, phenylalanine

hydroxylase, dopamine-β-hydroxylase and catechol-O-methyltransferase will be continued. The possible significance of false transmitters for histamine and serotonin will be explored. An attempt to rationalize the kinetic interactions of COMT, substrate, metabolism and S-adenosylmethionine in terms of a reaction mechanism and the formation of an active methyl-enzyme intermediate is in progress. The interactions of thio analogs of phenolic and catecholic biogenic amines with a variety of enzymes and biological systems will be explored in greater detail. Correlations between covalent binding of catecholamines and tryptamines to proteins and their long term cytotoxicity will be probed. Antibodies to COMT will be utilized to investigate the distribution of COMT-containing nerve networks in the central nervous system and the alteration of these networks by physiological, pharmacological and classical neurosurgical techniques. The fine localization of COMT with respect to both soluble and membrane-bound forms of the enzyme will be studied by immunofluorescent techniques and by peroxidase-coupled electron microscopy. The incorporation of fluorohistidines into protein will be studied with in vitro systems for protein synthesis.

Honors and Awards: None

Publications:

Creveling, C. R., Borchardt, R., and Isersky, C.: Immunological characterization of catechol-O-methyltransferase. In Snyder, S. H. and Usdin, E. (Eds.). Frontiers in Catecholamine Research. New York, Pergamon Press, 1974, pp. 117-119.

Daly, J., Fuxe, K., and Jonsson, G.: 5,7-Dihydroxytryptamine as a tool for the morphological and functional analysis of central 5-hydroxytryptamine neurons. Res. Commum. Chem. Path. Pharmacol. 7: 175-188, 1974.

Borchardt, R. T., Cheng, C.-F., Cooke, P. H., and Creveling, C. R.: The purification and kinetic properties of liver microsomal-catechol-O-methyltransferase. Life Sciences 14: 1089-1100, 1974.

Daly, J., Lundstrom, J., and Creveling, C. R.: Structure-activity correlations in trihydroxyphenethylamines and dihydroxytryptamines. Relationship to cytotoxicity in adrenergic and serotonergic neurons. In Fuxe, K., Olsen, J., and Zotterman, A. (Eds.). Wenner Gren Symposium XXII, "Dynamics of Degeneration and Growth in Neurons". New York, Pergamon Press, in press.

The Laboratory of Experimental Pathology experienced several changes during the past year which are now completed. The Section on Rheumatic Diseases which for the past 20 years represented an active research ar was abolished after Dr. Sokoloff's decision to join the Department of Pathology at Stony Brook where he continues to pursue his studies related to the pathogenesis of degenerative joint diseases. The investigations under way at the time of his departure have been carried to completion by Dr. Aubrey Hough, a member of Dr. Sokoloff's previous staff, in a most conscientious manner. The vacancy in the Section on Chemical Pathology, created in September 1972 when Dr. Marchesi, Chief of the Section, left for Yale University, was filled after a long search when Dr. Robert Friedman agreed to accept the position of Chief of the reconstituted Section. Dr. Friedman and his group will be largely involved in studies of virus-cell-interactions, a research area not previously pursued in this laboratory. A more detailed description of these research activities which have commenced will be given below.

Proteolytic Enzymes (Dr. Glenner and staff)

A stable pyrrolidonecarboxylate peptidase: A stable pyrrolidonecarboxylate peptidase (EC 3.4.11.8) was purified from Klebsiella cloacae. It was demonstrated that the purified enzyme liberates the pyrrolidonecarboxylyl moiety from proteins, oligopeptides and dipeptides, including L-pyrrolidonecarboxylyl-L-proline, oligopeptides and proteins. Enzyme was purified by Sephadex LH-20 chromatography, ammonium sulfate precipitation, DE-52 cellulose chromatography, removal of contaminating proteins by denaturation at -12°, Sephadex G-150 gel filtration and isoelectric focusing. Polyacrylamide gel electrophoresis revealed one protein band following the last purification step. The properties of the purified enzyme, such as its stability, pH optimum (7.0), molecular weight (74,200 daltons), Michaelis constant (0.4 mM), specificity for pyrrolidonylcarboxylate proteins have been determined. A new, water soluble chromogenic substrate, p(L-pyrrolidin-2-one-5-carboxamido) benzoic acid (PCA-PABA) was synthesized and used for the enzyme assay. A new method for the identification of pyrrolidonecarboxylic acid released from peptides and proteins dependent upon its hydrolytic conversion into glutamic acid is also described.

To our knowledge this is the first PCA-peptidase capable of acting on intact immunoglobulin proteins in addition to a wide variety of oligopeptides and dipeptides and of hydrolyzing a PCA-Pro peptide. These capabilities, therefore, extend the range of usefulness of the enzyme both as an analytical reagent and for unblocking PCA peptides previously inaccessible to automatic amino acid sequence analysis. No contaminating aminopeptidase, deacetylase, carboxypeptidase or endopeptidase activity in the purified enzyme preparation was detected. Our results indicated that the K. cloacae PCA-peptidase is an enzyme preferentially acting on short chain PCA-peptides.

The purified, highly stable PCA-peptidase can be completely coupled

to cyanogen bromide activated agarose, when the concentration of enzyme is 100 units/ml. The activity retained after coupling was 30% toward PCA-alanine and 70% toward PCA-paraaminobenzoic acid, respectively. The immobilized enzyme has been used repeatedly in column form without significant loss of activity. Experiments are in progress to use this enzyme to obtain total hydrolysis with high yields of PCA from amino terminus of such proteins as immunoglobulin heavy and lambda light chains. (Drs. Kwiatkowska, Glenner, Sipe and Mrs. Torain)

Amyloid Fibril Proteins

An amyloid fibril protein of unknown origin (AUO): Continuing studies of an AUO protein most frequently encountered in cases of amyloidosis defined clinically as being associated with another disease process ("secondary amyloidosis") show that a protein is present in the serum of these patients having antigenic determinants in common with the amyloid fibril protein. This protein (SAUO) migrates to the α - region on immunoelectrophoresis and is not antigenically related to any known serum protein, SAUO has not been found in normal serum nor in all cases of "primary" amyloidosis, but is present in 71% of patients with Familial Mediterranean Fever, a disease always associated with amyloidosis.

The tissue amyloid fibril protein (TAUO) has not been found in any case of myeloma associated amyloidosis or in cases of "primary" amyloidosis associated with excretion in the urine of an immunoglobulin light polypeptide chain. These findings confirm the results of our initial studies that one large group of systemic amyloidoses have amyloid fibrils which are composed almost entirely of a homogeneous light chain protein. (Drs. Glenner, Linke, Pollock, and Ignaczak).

Cytogenetics (Dr. Tjio and staff)

Meiotic and other studies of mouse hydrids: During the past several years, studies of the characteristics of mouse fetuses and newborn mice with trisomy for chromosome number 19 (the smallest autosome) were completed. Trisomic mice were produced by crossing mice carrying two different Robertsonian translocations (T1Wh plus T163H) with other strains of mice. Significant effects of trisomy 19 upon body weight were documented; placental weight also decreased, but to a less significant degree. Cleft palate and excessive degeneration of fetal oocytes were also associated with trisomy. Genetic background appeared to influence the appearance of trisomy-associated defects.

Previous reports from this laboratory described reduction of the mouse chromosome number from 40 to 38 and then from 38 to 36 by crossing mice with specific Robertsonian translocations. The strain with 36 chromosomes established several years ago (T1A1d/T1A1d T1Wh/T1Wh) has developed no abnormal characteristics. During the past year, it was crossed with another strain (T1IEM) to produce a new strain with 34 chromosomes. The new strain is now in the second generation, and it is too early to determine if it will be as normal as the parent strains. During the next year, we plan to observe litter size and meiotic chromosomes of these mice and their F_1 hybrids in order to determine if the further reduction in chromosome number affects repro-

ductive ability or frequency of meiotic nondisjunction. This is part of a long-term study of the genetic and evolutionary significance of Robertsonian translocations. (Dr. White and Tjio).

Studies on the origins of the cells which repopulate implanted splenic tissue of non-irradiated mice: It has been observed that autotransplantation of thymus, lymphnode and bone marrow at ectopic sites in experimental animals resulted in reorganization (after total necrosis) into structures that are histologically indistinguishable from the original organ. Similar findings were also observed when splenic tissue fragments were ectopically autotransplanted in mice, rats, and rabbits after total splenectomy. These tissues also underwent total necrosis followed by reorganization of the fragments into microscopically similar splenic fragments within eight weeks. Our studies, done in collaboration with Dr. V. V. Likhite, Harvard Medical Unit, Boston City Hospital, were formulated in order to elucidate the origin of the cells populating the explanted splenic tissue.

To evaluate whether these cells originated from the host or the donor, syngeneic DGA/2 male and female mice were used. After splenectomizing the host, the middle splenic fragment from the animal of the opposite sex was placed in one of the subcutaneous pockets in the left lateral thoraco-axillary region. At various periods after transplantation splenic implants were found to be from the host. Cultures made from the matrix of the implants exhibited the donor karyotype. In another experiment carried out with Dr. Brecher, San Franciso, with irradiated hosts, the cells that populated the implant had donor karyotype. (Dr. Tjio).

Control of Genetic Function in Normal and Virus-infected Animal Cells
(Dr. Friedman and staff)

Control in normal cells: One of the projects has been to study chromosomal arrangement and transcription of mammlian genes. The attempt is being made to purify specific mammalian cell mRNA species for use as molecular probes in analysis of gene structure and control of function. Preliminary studies indicate the feasibility of obtaining significant purification of particular mRNA species representing a very minor fraction of the total cellular mRNA.

The purified mRNA's will provide probes for use in various molecular hybridization procedures to study the chromosomal arrangement of specific genes and control of their transcription. Particular aspects of these studies will be aimed at determining if proteins containing two non-identical subunits (e.g., certain hormones) are specified by transcription of contiguous or non-contiguous genes and whether unbalanced synthesis of these subunits results from aberrant transcription or translation.

These studies will be expected to provide general procedures applicable to study of the molecular basis of many metabolic diseases resulting from absence of a protein. It should be possible to determine whether such metabolic diseases result from defects at the level of transcription or translation or from gene deletions or translocations. (Drs. Carter and Friedman)

Control in interferon-treated cells: Treatment of animal cells with the appropriate interferon induces changes in the cell which lead to the establishment of an antiviral state. Most viruses replicate poorly in such cells. Our plan is to study what changes interferon brings about and how virus replication is impaired in these cells.

Viral messenger RNA is translated with fidelity in an L cell system. In extracts from interferon-treated cells, the translation of viral messenger RNA is inhibited. Current work is directed toward the specificity of this inhibition and the possible interferon-induced factors involved. The understanding of the selective translational inhibition induced by interferon treatment may lead to a better understanding of post-transcriptional controls in animal cells.

Inhibition with interferon of RNA tumor virus induction by IUDR has also been achieved. It seems possible, however, that this is due to the inhibition of the replication of an induced entropic virus. We are currently attempting to design systems which will test possible inhibition in the actual induction phase of the process.

In addition, a substantial inhibition of virus production has been observed in AKR cells which spontaneously developed into C particle producing cultures. This was surprising because interferon has not usually been found effective in cells already virus-infected at the time of exposure to interferon. The implication of the work is that interferon may prove effective in controlling the replication of oncornaviruses (RNA tumor viruses). (Drs. Friedman, Pinkus, Carter, and Chang, and Mrs. Ramseur)

Control of virus-infected cells: Adeno-associated viruses (AAV) are defective parvoviruses. Because of the small size of their DNA genomes and unique mode of segregation of complementary DNA strands to separate virions, the AAV offer a unique situation amenable to biochemical analysis. In addition AAV infection is widespread in humans, and they are therefore important as potential human pathogens.

It has been found that a single 20S mRNA molecule is transcribed from the minus strand of the AAV genome. This RNA is equivalent to 70% of the length of the length of the DNA strand and has a molecular weight of 1×10^6 as determined in non-aqueous solvents such as DMSO or formamide. Molecular weight determinations of this RNA species in aqueous conditons yield aberrantly high values because of the presence of secondary structure or aggregation. The proportion (30%) of the minus strand of AAV DNA that is not represented in the stable 20S RNA transcript includes the 5' and 3' terminal inverted repetitions. There is no stable RNA synthesized from the AAV plus strand, but subsequent to its transcription in the cell nucleus, the 20S AAV messenger RNA has added to it a polyadenylate sequence of at least 90-100 nucleotides long. The 20S RNA is then transported from the nucleus to the polysomes without any post-transcriptional cleavage, and evidence to date strongly indicates that the 20S AAV RNA is probably monocistronic. The AAV genome therefore may contain only one gene.

Cleavage of AAV DNA with restriction endonuclease allows separation of left and right ends of the molecule. This together with subsequent separation

of the complementary strands of each DNA fragment allows further refinement of the transcription map of the AAV genome and more precise determination of the initiation and termination sites for transcription.

These studies are of interest because parvoviruses have been implicated in at least two human diseases: hepatitis A and a non-bacterial form of acute gastroenteritis. Our studies on AAV continue to be of major importance in laying a general framework for analysis of parvovirus biochemistry as a preliminary to an understanding of their role in human disease. Also, because AAV is defective and requires the presence of a second, helper virus (adenovirus or herpes viruses), these studies will provide further understanding of the basis of viral defectiveness and interference, both of which phenomena may play significant roles in viral diseases, Finally, since current evidence indicates that AAV genomes may contain only one gene, this system may offer a unique opportunity to study control of a single, specific gene in a mammalian cell. (Dr. Carter)

Additional studies will be undertaken in arborvirus-infected animal cells. These viruses are of special interest because they are associated with relatively simple structural outer membrane, and all of their synthetic processes (RNA and protein synthesis) are carried out on specific virus induced membrane structures. Studies on these viruses may provide valuable information on membrane structure and function. In addition, these agents go through a highly cytopathic replication cycle in mammalian cells while at the same time undergoing a relatively benign replication process in insect (their vectors) cells. It will be of interest to try to determine which (if any) of the virus directed processes are the cause of the severe cytopathic changes seen in the mammalian cell cultures; or, more interestingly what moderates the virus infection in insect cultures. Finally, the nucleocapsid of these agents is an interesting, yet simple, structure. It consists of a single, infectious viral RNA and a single polypeptide. It is planned to study the polypeptide. It has the interesting properties of being a virus structural protein which is produced on membranes and yet ends on a cytoplasmic structure (the virus nucleocapsid) which is not membrane associated. It also displays a great avidity for its particular RNA species. (Drs. Friedman and Chang)

Histochemical and Ultrastructural Studies: Methods and Applications (Dr. Feder and staff)

Beta-glucuronidase as a cell marker: In chimeric (tetraparental) mice derived by fusing high-glucuronidase (C57BL) and low-glucuronidase (C3H) strains, the mosaicism of the liver can be made apparent by means of the histochemical stain for beta-glucuronidase (Mintz, Proc. Nat. Acad. Sci 69: 2032, 1971).

Fixation techniques have been worked out in this laboratory to make this approach applicable to other cell types. In some cell types (hepatocytes, intestinal absorptive cells, adrenal cortical cells, corneal epithelia cells, and others), the high- and low-glucuronidase cells are clearly distinguished in the chimeric mouse by the large difference in the intensity with which their cytoplasm is stained; among these cell types almost every cell can be assigned to either the high- or low-glucuronidase group. Thus it may be possible to work out cell origins in transplanted tissues and to reconstruct

cell migrations during embryonic development (with Dr. Mullen and Dr. Sidman).

Marking neurons for light and electron microscopy: A promising approach to understanding neural integration is the microscopic identification and examination of individual neurons after the neuron has been injected with a marker dye through an intracellular microelectrode. The application of this method is limited at present by the very weak fluorescence of the dye that is used (Procion Yellow). Work is in progress to develop a better dye. Specifically, two N-substituted naphthalimides derived from 4-aminonaphthalic anhydride-3,6-disulfonic acid have been prepared. Both are brilliantly fluorescent and very soluble in water, and both have the capacity to bind covalently to tissue proteins, either directly or through the mediation of an aldehyde fixative. (with Mr. Stewart)

Freeze-fracture analysis of nerve structure: The ultrastructure as it relates to the function of a variety of synapses has been studied by freeze-fracture methods. In the olfactory bulb, excitatory snyapses are characterized by a distinctive collection of intramembranous particles, whereas no such specialization is discerned in nearby inhibitory synapses. Similarly, in the cerebellar cortex, three types of excitatory synapse are characterized by membrane-associated particles, whereas no such specialization is consistently recognized in two types of inhibitory synapse. Other features of nerve tissue have also been studied by freeze-fracture methods. In the olfactory bulb, cerebellar cortex, and a cochlear nucleus, a distinctive feature of the astrocyte membrane was found: there are orthogonal arrays of tightly packed particles on the membranes of astrocytes and their processes, but not on neuronal or oligodendroglial membranes. In a freeze-fracture analysis of the neuromuscular junction, the presynaptic membrane contained striking double rows of large particles. On stimulation, neurotransmitter release was associated with the fusion of synaptic vesicles with the nerve membrane in the region adjacent to the double row of particles. In another study, some striking similarities were found between the subsurface cisterns of mouse cerebellar neurons and those of amphioxus muscle. Both subsurface cisterns have a similar appearance in thin sections; moreover, in freeze-fractured preparations there are in both cases aggregates of particles on the cytoplasmic half of the plasmalemma where the plasmalemma overlies the cistern; and apparently in both cases it is near the subsurface cistern that electrical excitation of the plasmalemma is communicated to intracellular systems. (Drs. Landis, Reese, and associates)

Protein-Lipid Interactions in Lipoproteins of Membranes and Blood Plasma

The investigations described here, which terminate in this laboratory with this report, have been concerned with the topomolecular nature of the interactions that occur between the protein and lipid moieties of lipoproteins. The MN-glycoprotein (glycophorin), the major surface glycoprotein of the human erythrocyte, has been used as a model to study protein-lipid interactions as they occur in biological membranes. Specifically, an insoluble tryptic peptide produced from this protein can be associated with hydrated phospholipid bilayers in an artificial membrane system and the properties of the complex formed studied by a number of physical techniques. It has been found under these conditions that (1) the peptide is tightly bound to the lipid vesicles, (2) the peptide is largely helical when bound (suggesting hydrophobic binding), (3)

the permeability of the phospholipid vesicles containing T(is) to ions and water is markedly increased over the vesicles alone (again suggesting a hydrophobic binding) and (4) the peptide T(is) forms a multimeric peptide structure within the hydrophobic center of these bilayers as seen by freeze-etch electron microscopy. The intact MN-glycoprotein can also be associated with bilayers and is being studied in parallel. In a related project, computer analysis has been used to examine the hydrophobic nature of the peptide T(is) relative to presumably nonmembrane binding polypeptide segments.

Investigations into the nature of protein-lipid interactions in the blood plasma lipoproteins were brought into the project with the formulation of a topomolecular model for these associations based upon the known amino acid sequence of the approteins Apo LP-Ala and Apo LP-Ser from very low density lipoprotein and Apo LP-Gln II from high density lipoprotein. The model predicts the location of phospholipid binding "amphipathic helical" regions in these three proteins. In collaboration with Jackson, Morrisett and Gotto from Baylor Medical School, this theory has received fairly extensive experimental support in all three proteins. Further, and independently of the Baylor group, a computer analysis has been made to determine the frequency of occurrence of amino acid sequences similar to those described in the plasma lipoproteins in all sequenced proteins. The results of this analysis support the "amphipathic" model; the plasma lipoproteins are incredibly rich in "amphipathic" sequences compared to other proteins. In addition, the table of "amphipathic helixes" generated by this analysis is being used to suggest proteins, other than the plasma lipoproteins, which might bind lipids. For example, several polypeptide hormones contain "amphipathic helical" regions. Further, one of these regions composes the major portion of a fragment of bovine growth hormone which has biological activity. These findings lead one to speculate that this "amphipathic" region may bind to the membrane phospholipids of growth hormone receptor cells and thus in some manner trigger a transmembrane signal. Another protein, amyloid A, which is also rich in "amphipathic" regions has been found to bind with great avidity to phospholipid on the basis of several analytical tests. This previously unknown finding, predicted on the basis of the computer generated "amphipathic helix" table, is especially interesting from the standpoint of the in vivo function of Amyloid A, which is presently unknown. (Dr. Segrest)

Altered Thyroid Morphology with Altered Functional States (Dr. Tice)

The morphology of freeze-fracture preparations of normal, hypophysectomized, and TSH-stimulated thyroid glands was investigated. Principal findings were: first, that the width of the tight junctions found at the lateral plasma membranes of thyroid epithelial cells varied with the functional state of the gland. The most probable explanation for this phenomenon appears to be a simple elastic deformation of tight junctional elements with changes in height of thyroid epithelial cells. Second, details of the structure of pseudopod membrane (and that of colloid droplets) appears to differ from that of normal apical plasma membrane, from which the pseudopod membrane may be derived. The reason for this difference is not known, but may be related to the ease with which pseudopod membranes undergo fusion in the course of colloid droplet formation. (with Dr. Wollman)

In other studies the degenerative effects of 6-hydroxydopamine and 5,7-dihydroxytryptamine on the adrenergic nerve endings were examined. Both drugs lead to similar ultrastructural changes in terminal adrenergic axons. The effects of these drugs on ultrastructure of the thyroid gland is under study. (with Dr. Creveling)

Finally, studies were carried out on the localization of procollagen and procollagen peptidase in cultured fibroblasts using immunocytochemical techniques. Procollagen was found to be present in rough-surfaced endoplasmic reticulum. In addition, both antigenic activities were observed in small peripheral vesicles in fibroblasts, from which procollagen monomer is probably secreted. (with Dr. L. Kohn)

Studies in Immunochemistry

Immunochemical studies at an applied as well as at a basic level were continued. In the former category, efforts were directed at examining whether immunological or immunopathological mechanisms operate in the chloramphenicol induced aplastic anemia and tissue damage. Rats were injected with chloramphenicol and its less effective but less toxic analogue, thiamphenicol and at various stages of drug treatment, the animals were examined for hypersensitivity reactions and for circulating antibodies by conventional immunological techniques. These as yet incomplete studies revealed that some but not all of the experimental animals receiving chloramphenicol showed evidence of mild anaphylaxis when challenged with the drug and their sera contained low levels of antibodies reacting with a protein-chloramphenicol conjugate isolated from the animals. No such reactions were found in the animals receiving thiamphenicol. In other studies, antihistamine antibodies were obtained by immunizing rabbits with a histamine-protein conjugate. These specific antibodies are being applied at tissue level in immunohistochemical studies to obtain information on the manifold biological functions of histamine. In addition, studies were continued with the bifunctional reagent, p,p'-difluoro-m,m'-dinitrodiphenyl sulfone, which was previously employed successfully for conjugating antibodies to ferritin and enzymes respectively. This reagent has been found to decrease the susceptibility of some proteins to proteolytic digestion, without significantly altering their native antigenic properties, primarily from steric factors resulting from the intramolecular cross bridges. (Dr. Ram)

Biology of Degenerative Joint Diseases

As a part of the study of the natural history of degenerative joint disease, 68 osteoarthritic femoral heads were studied by systematic gross, roentgenographic and light microscopic examination. A higher than expected incidence of secondary aseptic necrosis was found to complicate pre-existing osteoarthritis. Five cases illustrating these findings will be presented by Dr. Sokoloff at a symposium on osteoarthritis in England in September, 1974.

Due to discrepencies in values obtained from cell cultures of cartilage

specimens and their chemical analyses among various investigators, it was thought that contamination with extraneous tissues might be responsible for these variations. Serial histologic examination of dissected specimens submitted by 17 different investigators disclosed that contamination was, in fact, widespread and ranged up to 50% of cross-sectional area. Histological examination of samples is thus a desirable quality control measure prior to analytical procedures. This paper is in press in Connective Tissue Research.

The role of the human osteochondral junction in the pathogenesis of osteoarthritis has remained largely speculative. A comprehensive light and electron microscopic study of normal human junction from 6 to 68 years has revealed the development of a collagenous bridging structure between bone and cartilage in adults. These studies represent new information which may contribute to our basic understanding of osteoarthritis. They are being prepared for publication. (Dr. Sokoloff and Dr. Hough)

One of the most crippling manifestations of hemophilia is the arthropathy which universally afflicts those with a severe deficiency of factor VIII. Light and electron microscopic studies of the articular cartilage in the earlier stages of this disease have revealed large amounts of iron localized to the chrondrocytes themselves. This phenomenon, which is not seen in states of systemic iron overload in humans, may be related to the early loss of articular cartilage in hemophilic arthropathy. (Dr. Hough)

Biomechanics and Related Studies (Dr. McCutchen)

A theoretical study of weeping lubrication is pursued to find out the optimum properties for a weeping bearing material to see how nearly cartilage approximates the optimum. This involves working out approximate mathematical theory and solving the equations with the aid of a simple analog computer.

Consultation for people in various sections of the laboratory and outside on a wide range of engineering and physical topics is provided which includes the development of an improved monitor to detect chattered ultrathin sections prepared for electron microscopy [see original article by McCutchen, Tice and Luft in J. Ultrastructural Research, 45: 297-302,(1973)]. The improved model is being developed by W. G. Friauf of DRS and the cooperating engineer is D. Thweatt.

Cycasin and Related Compounds (Dr. Laqueur)

In a recently prepared review on the oncogenicity of naturally occurring glycoside cycasin and of its synthetic aglycone methylazoxymethylacetate it became obvious that information was not available about the *in vivo* metabolism of two precursor compounds involved in the synthetic process, i.e., 1,2-dimethylhydrazine and azoxymethane. Although both compounds have in the meantime been shown to possess inherent oncogenic properties particularly with respect to colon-rectal cancer in conventional animals, no information is available whether similar effects can be observed in germfree animals. Procedures have been started to compare the effects of the two compounds in germfree rats after intragastric and parenteral administrations and to examine quantitatively the excretion patterns in urine and feces of the changed and unchanged compounds in a preliminary phase of this study. The experimental arrangements will be similar to those originally employed in our studies with cycasin and MAM acetate and comparison of the results will be possible.

Serial No. NIAMDD-LEP-1
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Metabolic and carcinogenic effects of Cycas circinalis, its glucoside cycasin and its aglycone, methylazoxymethanol, in conventional and germfree rats.

Previous Serial Number: Same

Principal Investigator: Gert L. Laqueur

Other Investigators: Dr. M. Spatz, Dr. H. Matsumoto, and Mr. E. G. McDaniel

Cooperating Units: LNNS-NINDS (Dr. Spatz)
LNE-NIAMDD (Mr. McDaniels)
University of Hawaii (Dr. Matsumoto)

Man Years:

Total: 4
Professional: 1
Other: 3

Project Description:

In the course of writing a review paper on the oncogenecity of the naturally occurring glucoside cycasin and its synthetic aglycone, methylazoxymethyl acetate (MAM acetate), it was pointed out that several precursor compounds of the synthetic MAM acetate, specifically 1,2-dimethylhydrazine and azoxymethane, have recently shown themselves to possess ongogenic properties. The organ system involved was the intestinal tract, in particular the colon and rectum. The final compound, MAM acetate, evidences carcinogenic effects in other organs as well, such as liver and kidney. The striking oncogenic effect of the two precursor compounds on the large bowel mucosa has led to their widespread use in developing animal models for studying the sequential mucosal changes leading to neoplasia and for examining the importance of a large number of factors contributing and controlling neoplastic transformation.

In spite of their extensive uses, very little is known of the fate of these two compounds in vivo. As a first approach to examining this question, we have decided to use germfree rats and administer by stomach tube as well as parenterally the two compounds and measure their appearance in urine and feces over a period of time. The project, which presently is undergoing preliminary methodological studies, is jointly conducted with Mr. McDaniels of LNE, NIAMDD, Dr. Matsumoto of the Agricultural Biochemistry Department, University of Hawaii,

and Dr. Yamamoto of CMT, NCI. Following the excretion studies, the germfree rats will be held over for later pathologic examination.

Publications:

None

Serial No. NIAMDD-LEP-2
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Preparation of stained tissue sections for investigation and diagnostic purposes.

Previous Serial Number: Same

Principal Investigator: Miss Virginia Reveal - Head, Tissue Preparation Laboratory

Other Investigators: None

Man Years:

Total: 2
Professional: 0
Other: 2

Project Description:

The statistical report of this unit is shown below.

	Specimen Accessioned	Stained Slides Routine	Stained Slides Special	Spare Slides	Total
Animals	1007	781			
Surgical	189	402			
		1183	7682		
		8865		7821	16,686

Serial No. NIAMDD-LEP-3
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Protein-protein interactions and immune processes in normal and disease states.

Previous Serial Number: Same

Principal Investigator: J. Sri Ram, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Immunological Studies on the Covalent Binding of Drugs to Tissue Macromolecules In Vivo: (Chloramphenicol and Thiamphenicol): An often encountered fall-out from treatment of humans with certain drugs is tissue damage as exemplified by pulmonary, hepatic, and renal necrosis, bone marrow depression, aplasia, and certain types of hypersensitivity reactions. One of the suggested causes for these undesirable side reactions of drugs is a possible modification of tissue macromolecules by the drug. This modification may take the form of denaturation of the tissue proteins or chemical alterations from the covalent binding of the drug or its metabolite to the protein, leading to a variety of pathological consequences. A similar mechanism has been proposed for the carcinogenicity of various chemicals. The clinical use of chloramphenicol, an effective broad spectrum antibiotic, has suffered a set back because of reports of aplastic anemia in some individuals who received the drug. A relatively less popular analog of this drug, namely, thiamphenicol, obtained by replacement of nitro group by a methylsulfonyl group was reported to produce no anemia in contrast to chloramphenicol. Preliminary studies carried out in NHLI indicated that both the drugs covalently bind to tissue proteins. It has been possible to isolate the drug-protein conjugate as well as the amino acid-drug conjugate following the hydrolysis of the former. It was decided to investigate if chloramphenicol produces its toxic effects via an immunological mechanism.

Male, Sprague-Dawley rats were injected with the drugs i.p. (30-100 mg/kg). Some animals received multiple injections one month apart. Each animal was

killed 24 hrs after the last injection and blood and various tissues were collected. The animals that received multiple injections were observed with each injection for symptoms of anaphylaxis. Some animals were also skin tested for cutaneous hypersensitivity reactions. The sera isolated from blood were studied by the following techniques: electrophoresis, gel diffusion reactions against the chloramphenicol-protein conjugate, and complement fixation. Three of six rats receiving chloramphenicol showed mild symptoms of anaphylaxis during multiple injections of the drugs. Two of six rats showed faint bands in immunodiffusion studies. On the other hand, none of the animals receiving thiamphenicol showed evidence of anaphylaxis, precipitin lines in gel diffusion, or complement fixation. Skin reactions were negative in all animals tested. These studies are being extended to longer drug treatment and to other animal species. Attempts are also underway to immunize rats with the isolated drug-protein conjugate to see if the symptoms of drug treatment can be reproduced.

Studies with the Bifunctional Reagent, p,p'-difluoro-m,m'dinitrophenylsulfone (FNPS): Previous studies on the chemical modification of bovine serum albumin with FNPS revealed that both intra- and intermolecular cross linkages are formed during the modification, without greatly altering the native hydrodynamic and antigenic properties. It was of interest to study how the formation of cross-linkages affects the susceptibility of the protein to various proteolytic enzymes, such as trypsin, chymotrypsin and pepsin, each with entirely different specificity requirements for peptide bond cleavage. Preliminary studies revealed that FNPS-modified bovine serum albumin exhibited near total resistance to digestion by all the three proteolytic enzymes. In view of the interest of this finding for providing a potential means of preparing soluble protein derivatives which resist intracellular digestion by proteolytic enzymes, these studies were extended to two other proteins, egg albumin and β-lactoglobulin. Accordingly, these proteins were reacted with FNPS under various conditions and the modified proteins were characterized. The kinetics of degradation of the modified derivatives by various proteolytic enzymes were compared with those of the native proteins. These studies confirmed the findings made with ovine serum albumin, that it is possible to prepare protein derivatives with greatly decreased susceptibility to proteolytic enzymes by chemical modification with FNPS. This resistance to proteolysis could not be attributed primarily to the decrease in lysyl and tyrosyl groups of the proteins but to the formation of the inter- and intramolecular cross linkages and the resulting steric effects.

Anti-Histamine Antibodies: Antibodies directed against the unique pharmacologic agent, histamine, were obtained employing as immunogen, protein carriers conjugated to histamine. The antibody levels were initially very low, making confirmation of antibody specificity ambiguous. Improved techniques with highly conjugated immunogens and prolonged and multiple immunizations have now yielded antibody levels allowing confirmation of the antibody specificity directed against histamine. Pure antibodies have been isolated using affinity chromatographic techniques. In experiments designed to throw light on the mechanisms of the manifold biologic roles of histamine, these antibodies will now be employed in immunohistochemical procedures at tissue

and cellular level in the anticipated new laboratory facilities permitting fluorescent microscopy.

Publications:

None

Serial No. NIAMDD-LEP-4
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Altered thyroid morphology with altered functional states

Previous Serial Number: NIAMDD-LEP-6

Principal Investigator: Lois W. Tice

Other Investigators: Dr. Seymour Wollman (LP-NCI)
Dr. C. R. Creveling (LC-NIAMDD)
Dr. L. Kohn (LBP-NIAMDD)

Man Years:

Total: 1.5
Professional: 1
Other: 0.5

Project Description:

The morphology of freeze-fracture preparations of normal, hypophysectomized, and TSH-stimulated thyroid glands was investigated. Principal findings were: first, that the width of the tight junctions found at the lateral plasma membranes of thyroid epithelial cells varied with the functional state of the gland. The most probable explanation for this phenomenon appears to be a simple elastic deformation of tight junctional elements with changes in height of thyroid epithelial cells. Second, details of the structure of pseudopod membrane (and that of colloid droplets) appears to differ from that of normal apical plasma membrane, from which the pseudopod membrane may be derived. The reason for this difference is not known, but may be related to the ease with which pseudopod membranes undergo fusion in the course of colloid droplet formation.

In other studies the degenerative effects of 6-hydroxydopamine and 5,7-dihydroxytryptamine on the adrenergic nerve endings were examined. Both drugs lead to similar ultrastructural changes in terminal adrenergic axons. The effects of these drugs on the ultrastructure of the thyroid gland is under study.

Finally, studies were carried out on the localization of procollagen and procollagen peptidase in cultured fibroblasts using immunocytochemical techniques. Procollagen was found to be present in rough-surfaced endoplasmic reticulum. In addition, both antigenic activities were observed in small peripheral vesicles in fibroblasts, from which procollagen monomer is probably secreted.

Publications:

McCutchen, C. W., Tice. L. W., and Luft, J. H.: Chatterbox: A vibration monitor to detect chattered sections at the ultramicrotome. J. Ultrastruct. Res. 45: 297-302, 1973.

Tice, L. W. and Wollman, S. H.: Ultrastructural localization of peroxidase on pseudopods and other structures of the typical thyroid epithelial cell. Endocrinology 1974.

Serial No. NIAMDD-LEP-5
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biomechanics and related studies.

Previous Serial Number: None

Principal Investigator: Charles W. McCutchen

Other Investigators: None

Man Years:

Total: 0.5
Professional: 0.5
Other: 0

Project Description:

A theoretical study of weeping lubrication is in progress. I hope to work out what are the optimum properties for a weeping bearing material to see how closely cartilages' properties match the optimum values. This involves working out an approximate mathematical theory and solving the equations with a simple analog computer. Some simple experiments are being done as part of this program.

With Miss Reveal, I am looking at histologic sections of dog artery in hopes of understanding how the collagen, elastin, and smooth muscle, with their diverse mechanical properties, cooperate to give arteries their observed behavior.

Consultation is provided for people in various sections within LEP and without on engineering and physical topics, which include the development of an improved Chatterbox, a vibration detector to monitor section cutting by an ultramicrotome (McCutchen, Tice, and Luft, J. Ultrastructure Research 45: 297-302, 1973). The improved model is being developed by Delhi Thweatt under the direction of Walter S. Friauf.

Publications:

McCutchen, C. W., Tice, L. W., and Luft, John H.: Chatterbox: a vibration monitor to detect chattered sections at the ultramicrotome. J. Ultrastructure Research 45: 297-302, 1973.

Serial No. NIAMDD-LEP-6
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Pathogenesis of experimental arthritis and pathology of rheumatism.

Previous Serial Number: None

Principal Investigator: Aubrey J. Hough, Jr., M.D.

Other Investigators: Dr. Sokoloff, Dr. Bland, Dr. Banfield

Cooperating Units: State University of New York at Stony Brook (Dr. Sokoloff)
University of Vermont (Dr. Bland)
NCI-LP (Dr. Banfield)

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Objectives:

1) Investigation of factors influencing development of degenerative joint disease in experimental animals and man.

2) Extending descriptive pathology of human rheumatic disease.

Methods Employed:

1) Articular and osseous tissues of humans and experimental animals are examined by light, scanning electron and transmission electron microscopy.

2) Elemental composition of specific anatomic features of joint tissues are performed by energy dispersive analysis of x-rays.

Major Findings:

1) A survey of a series of 68 osteoarthritic femoral heads systematically studied by gross, roentgenographic and light microscopic examination disclosed a higher incidence of aseptic necrosis of bone complicating preexisting osteoarthritis than had been anticipated. Five examples of this process will be presented at a Symposium on the pathology of osteoarthritis in England in September, 1974.

2) Twenty-two specimens of cartilage used for various experimental studies were examined by serial histological sectioning. Seventeen were contributed by investigators; 5 were obtained from a commercial supplier. The specimens comprised a spectrum of species and types of cartilage ranging from chick embryo sternum to bovine nasal septum. Eighteen of the 22 contained extraneous noncartilaginous tissue. The degree of contamination varied from 3 to 50% of the cross-sectional area. Calcification and vascularization are inherent anatomical features of several sorts of extra-articular cartilage. The impact of the contamination depends on the uses being made of the material and on the extent and specific activity of the extraneous component. It probably is greatest in cell culture studies because chondrocytes and fibroblasts have large differences in population doubling time. Histological examination of specimen material is thus a desirable quality control procedure in the design and interpretation of experiments on cartilage.

3) Combined light, scanning and transmission electron microscopy, and energy dispersive analysis of the articular osteochondral junctions of humans ranging in age from 6 to 68 years revealed that collagen fibers cross the junction itself, a heretofore unrecognized phenomenon. Energy dispersive analysis demonstrated significantly less calcium in the calcified cartilage than in the underlying bone. Osteochondral junctions of mature mice, rabbits, and guinea pigs resembled those of children, while those of adult monkeys and dogs resembled those of mature humans. A paper describing these findings is in the final stages of preparation.

4) Human hemophilic arthropathy has been studied by the techniques employed in (3). Iron is found in large amounts in the cartilage in early hemophilic arthropathy. This iron is localized to the cytoplasm of the original joint cartilage where it is located in membrane bound packets. No iron is identified by x-ray analysis in the extracellular matrix. This phenomenon may be responsible for the profound and early loss of cartilage in hemophilic arthropathy.

5) Osteoarthritic cervical spines have been examined microscopically for evidence of involvement of Luschka joints in the process.

6) Electron microscopic studies on the osteoarthritis of STR/IN mice will be performed prior to June 30, 1974.

Proposed Course:

The projects discussed above will have been completed prior to June 30, 1974 at which time the work of the section will be terminated. The senior investigator, Leon Sokoloff, M.D., retired June 15, 1973, and the remaining investigator, Aubrey Hough, M.D., is leaving active duty June 30, 1974.

Publications:

Hough, A. J., Mottram, F. C., and Sokoloff, L.: The collagenous nature of amianthoid degeneration of human costal cartilage. Amer. J. Path. 73: 201-216, 1973.

Srivastava, V. M. L., Malemud, C. M., Hough, A. J., Bland, J. H., Sokoloff, L.: Preliminary experience with cell culture of human articular chondrocytes. Arth. Rheum. 17: 165-169, 1974.

Hough, A. J. and Sokoloff, L.: Tissue sampling as a potential source of error in experimental studies of cartilage. Connec. Tissue Res. in press.

Malemud, C. M. and Sokoloff, L.: Some biologic characteristics of a pituitary growth factor (CGF) for articular chondrocytes in vitro. J. Cell Phys. in press.

Srivastava, V. M. L., Malemud, C. M., and Sokoloff, L: Chondroid expression by lapine articular chondrocytes in spinner culture following monolayer growth. Connec. Tissue Res. in press.

Serial No. NIAMDD-LEP-7
1. Experimental Pathology
2. Office of the Chief
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Protein-lipid interactions in lipoproteins of membranes and blood plasma

Previous Serial Number: None

Principal Investigator: Jere P. Segrest

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

The investigations described here, which terminate in this laboratory with this report, have been concerned with the topomolecular nature of the interactions that occur between the protein and lipid moieties of lipoproteins. The MN-glycoprotein (glycophorin), the major surface glycoprotein of the human erythrocyte, has been used as a model to study protein-lipid interactions as they occur in biological membranes. Specifically, an insoluble tryptic peptide produced from this protein can be associated with hydrated phospholipid bilayers in an artificial membrane system, and the properties of the complex formed studied by a number of physical techniques. It has been found under these conditions that (1) the peptide is tightly bound to the lipid vesicles, (2) the peptide is largely helical when bound (suggesting hydrophobic binding), (3) the permeability of the phospholipid vesicles containing T(is) to ions and water is markedly increased over the vesicles alone (again suggesting a hydrophobic binding) and (4) the peptide T(is) forms a multimeric peptide structure within the hydrophobic center of these bilayers as seen by freeze-etch electron microscopy. The intact MN-glycoprotein can also be associated with bilayers and is being studied in parallel. In a related project, computer analysis has been used to examine the hydrophobic nature of the peptide T(is) relative to presumably nonmembrane binding polypeptide segments.

Investigations into the nature of protein-lipid interactions in the blood plasma lipoproteins were brought into the project with the formulation of a topomolecular model for these associations based upon the known amino acid sequence of the apoproteins Apo LP-Ala and Apo LP-Ser from very low density

lipoprotein and Apo LP-Gln II from high density lipoprotein. The model predicts the location of phospholipid binding "amphipathic helical" regions in these three proteins. In collaboration with Jackson, Morrisett and Gotto from Baylor Medical School, this theory has received fairly extensive experimental support in all three proteins. Further, and independently of the Baylor group, a computer analysis has been made to determine the frequency of occurrence of amino acid sequences similar to those described in the plasma lipoproteins in all sequenced proteins. The results of this analysis support the "amphipathic" model; the plasma lipoproteins are incredibly rich in "amphipathic" sequences compared to other proteins. In addition, the table of "amphipathic helixes" generated by this analysis is being used to suggest proteins, other than the plasma lipoproteins, which might bind lipids. For example, several polypeptide hormones contain "amphipathic helical" regions. Further, one of these regions composes the major portion of a fragment of bovine growth hormone which has biological activity. These findings lead one to speculate that this "amphipathic" region may bind to the membrane phospholipids of growth hormone receptor cells and thus in some manner trigger a transmembrane signal. Another protein, amyloid A, which is also rich in "amphipathic" regions has been found to bind with great avidity to phospholipid on the basis of several analytical tests. This previously unknown finding, predicted on the basis of the computer generated "amphipathic helix" table, is especially interesting from the standpoint of the in vivo function of Amyloid A, which is presently unknown.

Publications:

Segrest, J. P., Kahane, I., Jackson, R. L., and Marchesi, V. T.: Major glycoprotein of the human erythrocyte membrane: evidence for an amphipathic molecular structure. Arch. Biochem. Biophys. 155: 167-183, 1973.

Segrest, J. P., Jackson, R. L., Marchesi, V. T., Guyer, R. B., and Terry, W.: Red cell membrane glycoprotein: amino acid sequence of an intramembranous region. Biochem. Biophys. Res. Commun. 49: 964-969, 1972.

Segrest, J. P. and Kohn, L. D.: Protein-lipid interactions of the membrane-penetrating MN-glycoprotein from the human erythrocyte. In Peeters, H. (ed.): Protides of the Biological Fluids - 21st Collaquium. Oxford and New York, Pergamon Press, 1974, pp. 183-189.

Segrest, J. P., Jackson, R. L., Morrisett, J. D., and Gotto, A. M.: A molecular theory of lipid-protein interactions in the plasma lipoproteins. FEBS Letters 38: 247-253, 1974.

Segrest, J. P., Gulik-Krzywicki, T., and Sardet, C.: Association of the membrane-penetrating polypeptide segment of the human erythrocyte MN-glycoprotein with phospholipid bilayers: I. Formation of freeze-etch intramembranous particles. Proc. Nat. Acad. Sci. U.S.A. in press.

Segrest, J. P. and Feldmann, R. F.: Membrane proteins: amino acid sequence and membrane penetration. J. Molec. Biol. in press.

Serial No. NIAMDD-LEP-8
1. Experimental Pathology
2. Chemical Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Control of genetic function in normal and virus-infected animal cells.

Previous Serial Number: None

Principal Investigators: Drs. Robert Friedman, Barrie J. Carter, Lawrence Pinkus, and Esther Chang

Cooperating Units: LP, NCI - Dr. A. Rabson
ARB, NIAMDD - Dr. C. Isersky
LBV, NIAID - Dr. G. Khoury
McGill University - Dr. T. Denhardt
Yale University - Dr. D. Ward

Man Years:

Total: 8
Professional: 4
Other: 4

Project Description:

I. Control of genetic function in normal cells.

A. Chromosomal arrangement and transcription of mammalian cell genes. (Drs. Carter and Friedman)

1. Objective

To purify specific mammalian cell mRNA species for use as molecular probes in analysis of gene structure and control of function.

2. Procedures

Human cells are grown in cell culture. Polysomes, as a source of mRNA, are prepared by ultracentrifugation of cell extracts. Messenger RNA species coding for particular proteins are purified from polysomes with the aid of specific antibodies and standard methods of nucleic acid fractionation, ultracentrifugation, electrophoresis and affinity chromatography.

3. Major findings and significance

This project has been recently established. Preliminary studies indicate the feasibility of obtaining significant purification of particular mRNA species representing a very minor fraction of the total cellular mRNA.

The purified mRNA's will provide probes for use in various molecular hybridization procedures to study the chromosomal arrangement of specific genes and control of their transcription. Particular aspects of these studies will be aimed at determining if proteins containing two non-identical subunits (e.g., certain hormones) are specified by transcription of contiguous or non-contiguous genes and whether unbalanced synthesis of these subunits results from aberrant transcription or translation.

These studies will be expected to provide general procedures applicable to study of the molecular basis of many metabolic diseases resulting from absence of a protein. It should be possible to determine whether such metabolic diseases result from defects at the level of transcription or translation or from gene deletions or translocations.

II. Control of genetic function in interferon-treated cells. (Drs. Friedman, Pinkus, Carter, and Chang, and Mrs. Ramseur)

A. Objectives

Treatment of animal cells with the appropriate interferon induces changes in the cell which lead to the establishment of an antiviral state. Most viruses replicate poorly in such cells. Our plan is to study what changes interferon brings about and how virus replication is impaired in these cells.

B. Procedures

1. Translation of virus specific and cellular messenger RNA's is being studied employing cell-free extracts from interferon-treated and control cells. Polypeptide synthesis is monitored in such systems and partial purification of the factors involved will be attempted.

2. Studies on the replication of RNA tumor viruses (oncornaviruses) are being carried out in interferon-treated mouse cells. In one system, virus induction from AKR and BALB/C cells by IUDR and other antimetabolites and antibiotics is under investigation; in another, virus replication in producer AKR cells is being studied.

3. Attempts are currently being made to develop a radioimmunoassay for direct analysis of binding of interferon to cells in culture. This procedure will then allow determination of whether the species specificity of interferon action is related to the specificity of

cell receptors. This system will also provide an additional probe for analysis of cell surface structure and function.

C. Major findings and significance

1. Inhibition of virus directed translation in extracts from interferon-treated cells.

Viral messenger RNA is translated with fidelity in an L cell system. In extracts from interferon-treated cells, the translation of viral messenger RNA is inhibited. Current work is directed toward the specificity of this inhibition and the possible interferon-induced factors involved. The understanding of the selective translational inhibition induced by interferon treatment may lead to a better understanding of post-transcriptional controls in animal cells.

2. Interferon inhibition of oncornaviruses.

Inhibition of RNA tumor virus induction by IUDR has been achieved. It seems possible, however, that this is due to the inhibition of the replication of an induced entropic virus. We are currently attempting to design systems which will test possible inhibition in the actual induction phase of the process.

In addition, we have observed a substantial inhibition of virus production in AKR cells which spontaneously developed into C particle producing cultures. This was surprising because interferon has not usually been found effective in cells already virus-infected at the time of exposure to interferon. The implication of the work is that interferon may prove effective in controlling the replication of oncornaviruses.

3. No significant findings have as yet been produced in this study.

III. Control of genetic function in virus-infected animal cells. (Drs. Carter, Chang, and Friedman)

A. Objectives of studies in AAV-infected cells (Dr. Carter)

Adeno-associated viruses (AAV) are defective parvoviruses. Because of the small size of their DNA genomes and unique mode of segregation of complementary DNA strands to separate virions, the AAV offer a unique situation amenable to biochemical analysis. In addition, AAV infection is widespread in humans, and they are therefore important as potential human pathogens. The objectives of this study are:

1. To analyse the structure of the AAV genome and obtain a transcription map.

2. To study structure and metabolism of AAV messenger RNA.

B. Procedures in studies in AAV-infected cells.

AAV is grown in human cells which are coinfected with a second helper (adenovirus) virus. Viral DNA and RNA are labeled by incorporation of radioactive precursors and purified by standard procedures. The conformation of RNA and DNA is analysed by ultracentrifugation, gel electrophoresis, molecular hybridization, affinity chromatography, and specific cleavage with restriction endonuclease.

C. Major findings in AAV-infected cells.

1. A single 20S mRNA molecule is transcribed from the minus strand of the AAV genome. This RNA is equivalent to 70% of the length of the DNA strand and has a molecular weight of 1×10^6 as determined in non-aqueous solvents such as DMSO or formamide. Molecular weight determinations of this RNA species in aqueous conditions yields aberrantly high values because of the presence of secondary structure or aggregation.

2. The proportion (30%) of the minus strand of AAV DNA that is not represented in the stable 20S RNA transcript includes the 5' and 3' terminal inverted repetitions. There is no stable RNA synthesized from the AAV plus strand.

3. Subsequent to its transcription in the cell nucleus, the 20S AAV RNA has added to it a polyadenylate sequence of at least 90-100 nucleotides long. The 20S RNA is then transported from the nucleus to the polysomes without any post-transcriptional cleavage.

4. Cleavage of AAV DNA with restriction endonuclease allows separation of left and right molecular ends of the molecule. This together with subsequent separation of the complementary strands of each DNA fragment allows further refinement of the transcription map of the AAV genome and more precise determination of the initiation and termination sites for transcription.

5. Evidence to date strongly indicates that the 20S AAV RNA is probably monocistronic and that the AAV genome therefore may contain only one gene.

D. Significance of AAV studies.

1. Recently, parvoviruses have been implicated in at least two human diseases: hepatitis A and a non-bacterial form of acute gastroenteritis. Our studies on AAV continue to be of major importance in laying a general framework for analysis of parvovirus biochemistry as a preliminary to an understanding of their role in human disease.

2. Because AAV is defective and requires the presence of a second, helper virus (adenovirus or herpes viruses), these studies will provide further understanding of the basis of viral defectiveness and interference, both of which phenomena may play significant roles in viral diseases.

3. Current evidence indicates that AAV genomes may contain only o. gene. This system thus offers a unique situation to study control of a single, specific gene in a mammalian cell.

E. Projected studies in arbovirus-infected cells. (Drs. Chang and Friedman)

Additional studies will be undertaken in arbovirus-infected animal cells. These viruses are of special interest for several reasons.

1. They are associated with a relatively simple structural outer membrane, and all of their synthetic processes (RNA and protein synthesis) are carried out on specific virus induced membrane structures. Studies on these viruses may provide valuable information on membrane structure and function.

2. These agents go through a highly cytopathic replication cycle in mammalian cells while at the same time undergoing a relatively benign replication process in insect (their vectors) cells. It will be of interest to try to determine which (if any) of the virus directed processes are the cause of the severe cytopathic changes seen in the mammalian cell cultures; or, more interestingly, what moderates the virus infection in insect cultures.

3. The nucleocapsid of these agents is an interesting, yet simple, structure. It consists of a single, infectious viral RNA and a single polypeptide. We hope to study the polypeptide. It has the interesting properties of being a virus structural protein which is produced on membranes and yet ends on a cytoplasmic structure (the virus nucleocapsid) which is not membrane associated. It also displays a great avidity for its particular RNA species.

Publications:

None

Serial No. NIAMDD-LEP-9
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Histochemistry: principles, methods, and applications

Previous Serial Number: NIAMDD-LEP-5

Principal Investigator: Ned Feder

Other Investigators: Richard Mullen, Richard L. Sidman, Walter W. Stewart

Cooperating Units: Harvard Medical School (Mullen, Sidman)
Harvard University (Stewart)

Man Years:

Total: 1.5
Professional: 1
Other: 0.5

Project Description:

Beta-glucuronidase as a cell marker: In chimeric (tetraparental) mice derived by fusing high-glucuronidase (C57BL) and low-glucuronidase (C3H) strains, the mosaicism of the liver can be made apparent by means of the histochemical stain for beta-glucuronidase (Mintz, Proc. Nat. Acad. Sci. 69: 2032, 1971). Fixation techniques have been worked out in this laboratory to make this approach applicable to other cell types. In some cell types (hepatocytes, intestinal absorptive cells, adrenal cortical cells, corneal epithelium cells, and others), the high- and low-glucuronidase cells are clearly distinguished in the chimeric mouse by the large difference in the intensity with which their cytoplasm is stained; among these cell types almost every cell can be assigned to either the high- or low-glucuronidase group. Thus it may be possible to work out cell origins in transplanted tissues and to reconstruct cell migrations during embryonic development (with Dr. Mullen and Dr. Sidman).

Marking neurons for light and electron microscopy: A promising approach to understanding neural integration is the microscopic identification and examination of individual neurons after the neuron has been injected with a marker dye through an intracellular microelectrode. The application of this method is limited at present by the very weak fluorescence of the dye that is used (Procion Yellow). Work is in progress to develop a better dye. Specifically, two N-substituted naphthalimides derived from 4-aminonaphthalic anhydride-3,6-disulfonic acid have been prepared. Both are brilliantly fluorescent and very soluble in water, and both have the capacity to bind covalently to tissue proteins, either directly or through the mediation of an

aldehyde fixative (with Mr. Stewart).

Publications:

None

Serial No. NIAMDD-LEP-10
1. Experimental Pathology
2. Biophysical Histology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Freeze-fracture analyses of membrane structure in neurons, astrocytes, and at the neuromuscular junction

Previous Serial Number: NIAMDD-LEP-7

Principal Investigator: Dennis M. D. Landis, M.D.

Other Investigators: T. S. Reese, M.D.
M. Henkart, Ph.D.
E. Raviola, M.D.

Cooperating Units: LNNS, NINDS
BB, NICHD
Department of Anatomy, Harvard Medical School

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Freeze-fracture analysis of synaptic structure in the olfactory bulb: The reciprocal dendro-dendritic synapse between granule cell gemmules and mitral cell dendrites in the external plexiform layer of the olfactory bulb has been studied with freeze-fracture techniques in an effort to compare synaptic membrane structure at contiguous inhibitory and excitatory contacts. The excitatory contact was characterized by specialization of intramembranous particle distribution in both presynaptic and postsynaptic membranes, while no intramembranous specializations could be discerned at the inhibitory contact. Gap junctions were discovered between proximal mitral dendrites and gemmules, a previously unrecognized feature of synaptic organization in the bulb.

Freeze-fracture analysis of synaptic structure in the cerebellar cortex: Membrane structure as revealed by freeze-fracture techniques at three excitatory synapses (parallel fiber-to-Purkinje spine, climbing fiber-to-Purkinje spine, mossy/climbing fiber-to-granule cell dendrite) was characterized by prominent particles on the cytoplasmic half of the presynaptic membrane, a widened synaptic cleft, and an aggregate of particles on the external half of the postsynaptic membrane. In striking contrast, no specializations of particle distribution could be consistently recognized at two inhibitory synapses (stellate cell axon-to-Purkinje dendrite, basket cell axon-to-Purkinje soma). Since

particles in freeze-fractured membranes probably represent membrane associated protein, the differences in structure observed likely reflect distinctive patterns of molecular organization at synapses of different physiological activity. Two classes of interaction between vesicles and the axonal plasmalemma were also studied which probably correspond to synaptic vesicle exocytosis and coated vesicle endocytosis.

Freeze-fracture study of astrocytes: The membrane characteristics of astrocytes were examined in the olfactory bulb, cerebellar cortex, and anteroventral cochlear nucleus of mice. Distinctive orthogonal arrays of tightly-packed particles (with a periodicity of 7-8nm) were uniformly present on the cytoplasmic half of astrocytic membranes forming perivascular sheaths and marginal processes. Fewer, but otherwise exactly similar, particle arrays were identified in perineuronal astrocytic processes in the neuropil. No comparable structures were ever encountered in neuronal or oligodendroglial membranes. It seems likely that these particle aggregates, which we term "assemblies", are astrocytic specializations which may be involved in governing the concentration of substances in the interstitial fluid of the brain.

Freeze-fracture analysis of neuromuscular junction structure: The morphology of the neuromuscular junction of the frog sartorius muscles was examined under various conditions of stimulation in several media in vitro. The presynaptic membrane of the freeze-fractured motor nerve terminal contained striking double rows of large particles which were coextensive with the presynaptic dense bands observed in thin sections. The postsynaptic sarcolemma was distinguished by prominent aggregates of large particles associated with the cytoplasmic membrane half. Neurotransmitter release was found to be associated with the fusion of synaptic vesicles with the nervem membrane at discrete points, adjacent to the double particle row, along the terminal. The number of synaptic vesicle fusion sites observed during transmitter release varied with the fixative employed. Intramembranous sarcolemma structure after denervation and after muscle section is currently under study.

Freeze-fracture study of subsurface cisterns in cerebellar neurons and amphioxus muscle: Subsurface cisterns are a specialization of endoplasmic reticulum in neuronal somata and proximal dendrites. Their structure in thin sections is remarkably similar to certain types of sarcoplasmic reticulum in muscle. In freeze-fractured preparations, neuronal subsurface cisterns were found to have a peculiar intramembranous structure, and the overlying neuronal plasmalemma manifested a striking aggregate of particles on its cytoplasmic half. Similarly, the sarcolemma of amphioxus muscle was found to contain aggregates of particles coextensive with underlying cisterns. The muscle subsurface cisterns almost certainly are sites where action potentials trigger calium release into the sarcoplasm. The morphological similarities suggest that neuronal subsurfact cisterns also function to relate membrane and cytoplasmic activity.

Freeze-fracture studies of unfixed, uncryoprotected cerebellar cortex: All freeze-fracture studies of central nervous system structure have hitherto employed tissue fixed in aldehydes and equilibrated with glycerol solutions

prior to freezing. Since both fixation and cryoprotection exert some effect on tissue structure, we have sought to eliminate these potential sources of artifact. The principal difficulty is that the rate of freezing obtained with conventional techniques is insufficient to prevent intracellular damage from ice crystals in non-cryoprotected tissue. We have successfully employed the apparatus originally described by Van Harreveld to freeze the superficial regions of mouse cerebellar cortex and are in the process of analyzing the structures encountered as compared to those present in tissue prepared by conventional methods.

Publications:

Landis, D. M. D., Henkart, M., Reese, T. S.: Similar arrays of plasma membrane particles at subsurface cisterns in striated muscle and neurons. J. Cell Biol. 59: 184A, 1973.

Heuser, J. E., Reese, T. S., Landis, D. M. D.: Functional changes in frog neuromuscular junctions studied with freeze-fracture. J. Neurocytol. 3: 109-131, 1974.

Landis, D. M. D., Reese, T. S.: Arrays of particles in freeze-fractured astrocytic membranes. J. Cell Biol. 60: 316-320, 1974.

Landis, D. M. D., Reese, T. S., Raviola, E.: Differences in membrane structure between excitatory and inhibitory components of the reciprocal synapses in the olfactory bulb. J. Comp. Neurol. in press.

Landis, D. M. D., Reese, T. S.: Differences in membrane structure between excitatory and inhibitory synapses in the cerebellar cortex. J. Comp. Neurol. in press.

Landis, D. M. D., Rosenberg, R. N., Landis, S. C., Schut, L., Nyhan, W. L.: Clinical and ultrastructural abnormalities in olivopontocerebellar degeneration. Archives of Neurology. in press.

Serial No. NIAMDD-LEP-11
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Protein and proteolytic enzyme interrelationships in normal and disease states.

Previous Serial Number: NIAMDD-LEP-9

Principal Investigator: George G. Glenner, M.D.

Other Investigators: J. Kwiatkowska, Ph.D.
R. P. Linke, M.D.
P. Scott Pollock, M.D.

Cooperating Units: NIDR-LHP (H. Bladen, Ph.D.)
NIDR-LHP (D. Eanes, Ph.D.)
NCI-I (W. Terry, M.D.)
NIDR-LHP (J. Termine, Ph.D.)

Man Years:

Total: 5 1/2
Professional: 4
Other: 1 1/2

Project Description:

Purification of a Pyrrolidonecarboxylate Peptidase: A stable "particle-bound" pyrrolidonecarboxylate peptidase (EC 3.4.11.8) was purified from Klebsiella cloacae. It was demonstrated that the purified enzyme liberates the pyrrolidonecarboxylyl moiety from proteins, oligopeptides and dipeptides, including L-pyrrolidone-carboxylyl-L-proline. The enzyme was isolated by means of sodium deoxycholate extraction and was purified by Sephadex LH-20 chromatography, ammonium sulfate precipitation, DE-52 cellulose chromatography, and removal of contaminating proteins by denaturation at -12°. Acrylamide gel electrophoresis revealed one protein band following the last purification step. The properties of the purified enzyme, such as its stability, pH optimum (7.0), molecular weight (74,200 daltons), Michaelis constant (0.4mM), and specificity for pyrrolidonyl proteins, have been determined. A new water soluble chromogenic substrate, p(L-pyrrolidin-2-one-5-carboxamido) benzoic acid was synthesized and used for the enzyme assay. A new method for the identification of pyrrolidonecarboxylic acid released from peptides and proteins dependent upon its hydrolytic coversion into glutamic acid is also described.

To our knowledge, this is the first PCA-peptidase capable of acting on intact immunoglobulin proteins (13) in addition to a wide variety of

oligopeptides and dipeptides. These capabilities, therefore, extend the range of usefulness of the enzyme both as an analytical reagent and for unblocking PCA peptides previously inaccessible to automatic amino acid sequence analysis. No contaminating aminopeptidase, deacetylase, carboxypeptidase or endopeptidase activity was detected in the purified enzyme preparation. Our results indicate that the "particle-bound" K. cloacae PCA-peptidase is an enzyme preferentially acting on short chain PCA-peptides. This is a feature shared by all other PCA-peptidases reported. In an attempt to increase the total amount of PCA liberated from proteins within a reasonable experimental time period, we considered the possibility of increasing the enzyme-substrate ratio. In a soluble system, an increase of the enzyme-substrate ratio beyond a certain limit would, however, reduce the usefulness of the enzyme when applied for the purpose of removing the PCA blocking group from peptides or proteins in order to perform subsequent amino acid sequence analysis. We have, therefore, directed our attention to the alternative possibility that an immobilized enzyme would be ideal for such a purpose. The stability of the "particle-bound" PCA-peptidase further enhanced the feasibility of such a system. Immobilization of the active enzyme by cyanogen bromide coupling to Sepharose 4B has succeeded in providing an increased enzyme-substrate ratio with a greater extent of hydrolysis in the absence of contamination of hydrolytic products by enzymic protein. A description of this solid-phase system will be the subject of a separate report.

The value of the substrate, PCA-PABA, resides in its absolute specificity for NH_2-terminal PCA hydrolysis by PCA-peptidase (as compared to e.g. PCA-Ala, which can be hydrolysed by carboxypepdidases), its aqueous solubility and the simplicity of its use in a colorimetric assay and consequent kinetic analysis of the enzyme. Szewczuk and Mulczyk reported the use of a potentially chromogenic substrate, L-pyrrolidonecarboxylyl β-naphthylamide, for estimation of PCA-peptidase activity, a substrate which could also have been employed in a flouorometric assay technique. The aqueous insolubility of this substrate, however, required the use of 10% methyl alcohol for solubilization. The deleterious effect of the solvent on the enzyme and the reported failure of the substrate to saturate the enzyme led us to the synthesis of PCA-PABA in order to eliminate these difficulties.

Amyloidosis: An amyloid fibril protein of unknown origin (AUO): Continuing studies of an AUO protein most frequently encountered in cases of amyloidosis, defined clinically as being associated with another disease process ("secondary amyloidosis"), show that a protein is present in the serum of those patients having antigenic determinants in common with the amyloid fibril protein. This protein (SAUO) migrates to the α_1 region on immunoelectrophoresis and is not antigenically related to any known serum protein. SAUO has not been found in normal serum nor in most cases of "primary" amyloidosis, but is present in 71% of patients with Familial Mediterranean Fever, a disease always associated with amyloidosis.

The tissue amyloid fibril protein (TAUO) has not been found in any case of myeloma associated amyloidosis or in cases of "primary" amyloidosis associated with excretion in the urine of an immunoglobulin light polypeptide chain.

These findings confirm the results of our initial studies that one large group of systemic amyloidosis have amyloid fibrils which are composed almost entirely of a homogeneous light chain protein.

Honors and Awards:

USPHS Meritorious Service Medal, 1973
Guest Lecturer: Saul R. Korey Memorial Symposium, Albert Einstein School of Medicine, New York, 1973
Necker's Seminars in Nephrology, Hôpital Necker, Paris, France, 1974

Publications:

Terry, W. D., Page, D. L., Kimura, S., Isobe, T., Osserman, E. F., and Glenner, G. G: Structural identity of Bence Jones and amyloid fibril proteins in a patient with plasma cell dyscrasia and amyloidosis. J. Clin. Invest. 52: 1276-1281, 1973.

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Glenner, G. G. and Terry, W. D.: The immunoglobulin origin of amyloid fibril proteins. In Peeters, H. (ed.): Protides of the Biological Fluids - 20th Colloquium. Brugge, Belgium, 1972, pp. 55-62.

Glenner, G. G.: Formation of pathological proteins. US - Japan Seminar. Acta Histochem. Cytochem 5: 278-279, 1972.

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Glenner, G. G. and Grimley, P. M.: Tumors of the extra-adrenal paraganglion system (including chemoreceptors). In Atlas of Tumor Pathology, Series 2, Fascicle 9. Washington, D.C., Armed Forces Institute of Pathology, 1974, 90 pp.

Glenner, G. G. and Terry, W. D.: Characterization of amyloid. Ann. Rev. Med. 25: 131-148, 1974.

Serial No. NIAMDD-LEP-12
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cytogenetics

Previous Serial Number: NIAMDD-LEP-10

Prinicpal Investigator: Dr. J. H. Tjio

Other Investigators: Dr. B. J. White

Cooperating Units: Maryland Psychiatric Research Center
NCI-LPHY (Dr. W. W. Smith)
University of California Medical Center, San Francisco (Dr. G. Brecher)
University of California, San Diego (Dr. G. Sato)
University of Colorado Medical Center, Denver (Dr. T. T. Puck)
Harvard Medical Unit, Boston City Hospital (Dr. V. V. Likhite)

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Cytogenetic studies on the effect of LSD: We are currently studying the effects of the hallucinogens LSD and DPT on human chromosomes. Several problems have arisen due to the death of Dr. W. N. Pahnke, the principal collaborator.

Chromosome identification and studies of chromosomal abnormalities in the mouse and human using differential staining techniques: Several strains of mice have been studied using Giemsa-banding, quinacrine mustard-banding and centromeric heterochromatin-staining. These three methods serve as a multiple genetic marker system for various cytogenetic studies. The quantitative variation of the centromeric heterochromatin among homologous chromosomes could be used as a marker for genetic studies.

Chromosome identification in cell fusion experiments: Chinese hamster ovary cells (grown by Dr. T. T. Puck, University of Colorado Medical Center, Denver) with a specific enzymatic deficiency which results in glycin auxotrophy were fused with human cells; the resulting hybrids were grown on selective

media to isolate those lines which had altered their glycine response. The chromosomes of these clones, and of the parent hamster line, are being examined in our laboratory.

Studies on the origins of the cells which repopulate implanted splenic tissue of non-irradiated mice: It has been observed that autotransplantation of thymus, lymph node and bone marrow at ectopic sites in experimental animals resulted in reorganization (after total necrosis) into structures that were histologically indistinguishable from the original organ. Similar findings were also observed when splenic tissue fragments were ectopically autotransplanted in mice, rats, and rabbits after total splenectomy. These tissues also underwent total necrosis followed by reorganization of the fragments into microscopically similar splenic fragments within eight weeks.

Our studies, done in collaboration with Dr. V. V. Likhite, Harvard Medical Unit, Boston City Hospital, were formulated in order to elucidate the origin of the cells populating the explanted splenic tissue.

To evaluate whether these cells originated from the host or the donor, syngeneic DBA/2 male and female mice were used. After splenectomizing the host, the middle splenic fragment from the animal of the opposite sex was placed in one of the subcutaneous pockets in the left lateral thoraco-axillary region. At various periods after transplantation, splenic implants were found to be from the host. Cultures made from the matrix of the implants exhibited the donor karyotype. In another experiment carried out with Dr. Brecher, San Francisco, with irradiated hosts, the cells that populated the implant had donor karyotype.

Due to the findings with Dr. Brecher, we will repeat the experiments in this study using immunocompromised DBA/2 mice.

Cytogenetic studies on two clonal lines of B16 melanoma: Cells were obtained from Dr. G. Sato, San Diego. This study was performed to find out if there are any systematic chromosomal differences between these two stains. One of these metastasizes extensively and the other does not. Thus far, no definite conclusions could be made due to difficulties in the techniques. A rat ovarian tumor culture 31A was also studied.

Clinical cytogenetic studies on human congenital and developmental disorders: The differential staining methods of quinacrine mustard fluorescence and Giemsa-banding are being applied in our laboratory to studies of patients with various genetic disorders, abnormalities of gonadal development and function, and selected cases of infertility in the male. Since in many such patients no gross chromosomal abnormalities are detectable using routine cytogenetic methods, the differential staining procedures provide a means to detect small structural changes in chromosomes. A rearrangement that does not produce an obvious change in chromosome morphology can often be identified by altered banding patterns in the chromosomes involved, such as the duplication or deletion of a chromosomal segment, peri-centric inversion, and some cases of reciprocal translocation. Since the Y chromosome has a characteristic

Giemsa-banding pattern, the differential-staining analysis of these chromosomes enables us to make a more precise diagnosis in many patients with abnormal gonadal development and function to determine whether the hybrids retain any human chromosomes, and, if so, which ones are involved. If any human chromosomes are present, analysis of several clones should permit the assignment of the enzyme in question to the chromosome responsible for its production, linkage studies and gene mapping could follow.

Pattern of recovery of hemopoiesis in the mouse after irradiation and and mechanical depopulation: In cooperation with Dr. George Brecher (San Francisco) and Dr. W. Smith (NCI), we have previously shown that in radiation chimeras in which AKR mice have hemopoietic cells entirely of T_1Ald karyotype, hemopoietic repopulation of mechanically depleted marrow is solely from T_1Ald cells. It had been established by others that the hemopoietic repopulation is of local origin and possibly due to a common precursor of bone and hemopoietic marrow cells. Our experiment appeared to rule out the existence of such a common precursor in repopulation after mechanical depletion. We are now engaged in repeating our experiment using a new method assuring complete depopulation of hemopoietic cells in the mouse femur followed by keryotyping of both the hemopoietic cells and the supportive stroma after culture.

The old observation that transfusion of syngeneic marrow takes only in irradiated, but not in unirradiated animals was further explored with the T_1Ald marker. Attempts to establish T_1Ald hemopoietic cells in unirradiated AKR recipients was unsuccessful even after phenylhydrazine and endotoxin. These durgs were used to put stem cells of either donor or recipient into cycle to insure that stem cells, which are normally not in cycle, will not simply remain dormant and escape detection in the recipient.

The T_1Ald-AKR system was used to determine the origin of hemopoietic cells in the omental grafts. Hemopoiesis was of donor origin at 10 days in irradiated, but of host origin in non-irradiated animals. At 35 days the implants in irradiated animals were of mixed host and donor origin. The cytogenetic findings point to the profound effect of radiation on transplantability of hemopoiesis even in the syngeneic situation.

We are now exploring the possible existence of minor histoincompatibility between T_1Ald and AKR. We will study the F_1 hybrids of the 2 strains, which will have one rather than two of the T_1Ald marker chromosomes. These strains will be used as either donors to both parental lines or as recipients from either parent. If histoincompatibility rather than the normal unirradiated state is the cause of the failure of takes, at least one of the combinations of transplants between F_1 and parental strains may succeed.

An additional alternative exists that the post-irradiation state is effective because it reduces the number of potential host precursors to less than 1 in 1,000 and thus provides optimal opportunity for donor cells to compete with any surviving host stem cells. This possibility is being explored by use of myleran which is known to reduce stem cells as represented by colony forming units similar to irradiation without being as effective as immuno-suppressant.

Honors and Awards:

Doctor Honoris Causa, University Claude Bernard, Lyon, France, 11 June, 1974.

Publications:

None

Serial No. NIAMDD-LEP-13
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cytogenetic studies

Previous Serial Number: NIAMDD-LEP-11

Principal Investigator: Dr. B. J. White

Other Investigators: Dr. J. H. Tjio

Cooperating Units: NIDR-HG (Dr. K. Brown)
NICHD-RR (Dr. J. Vaitukaitis)
NIDR-CP (Dr. E. Driscoll)
NINDS-PR (Dr. J. Sever)
NINDS-LEN (Dr. W. Caveness)
NIAID-LVD (Dr. M. Sklar)

Man Years:

Total: 2
Professional: 1
Other: 1

Project Description:

Cytogenetic and reproductive studies of mice with Robertsonian translocations and their hybrids.

Continued studies of the T1Wh strain: This strain, developed in our laboratory in 1967, is in generation 27 of inbreeding. The average litter size at birth has remained stable (5.9 at generation 26), and no abnormalities have appeared in the colony. Some of these mice have recently been shipped to several other laboratories conducting studies of mouse Robertsonian translocations and continue to be utilized for projects within our own laboratory.

Meiotic and other studies of mouse hybrids: During the past several years, studies of the characteristics of mouse fetuses and newborn mice with trisomy for chromosome number 19 (the smallest autosome) were completed. Trisomic mice were produced by crossing mice carrying two different Robertsonian translocations (T1Wh plus T163H) with other strains of mice. Significant effects of trisomy 19 upon body weight were documented; placental weight was also decreased, but to a less significant degree. Cleft palate and excessive degeneration of fetal oocytes were also associated with trisomy. Genetic background appeared to influence the appearance of trisomy-associated defects. Two manuscripts concerning these observations are currently in press. At present, we are collabor-

ating with Dr. K. Brown (NIDR-HG) in more detailed studies of cleft palate in trisomic fetuses. The studies emphasize the similarity between the effects of autosomal trisomy upon growth and development in humans and mice.

Previous reports from this laboratory described reduction of the mouse chromosome number from 40 to 38 and then from 38 to 36 by crossing mice with specific Robertsonian translocations. The strain with 36 chromosomes established several years ago (T1A1d/T1A1d T1Wh/T1Wh) has developed no abnormal characteristics. During the past year, it was crossed with another strain (T1IEM) to produce a new strain with 34 chromosomes. The new strain is now in the second generation, and it is too early to determine if it will be as normal as the parent strains. During the next year, we plan to observe litter size and meiotic chromosomes of these mice and their F_1 hybrids in order to determine if the further reduction in chromosome number affects reproductive ability or frequency of meiotic nondisjunction. This is part of a long-term study of the genetic and evolutionary significance of Robertsonian translocations.

Cytogenetic and dermatoglyphic studies of patients: A total of 36 patients with a variety of genetic or congenital abnormalities were karyotyped using chromosomal banding techniques. These patients included the following: primary amenorrhea, Noonan's syndrome, pseudohypoparathyroidism, short stature of unknown etiology, cerebral gigantism, Prader-Willi syndrome, isolated growth hormone deficiency, and X-linked vitamin D-resistant rickets. Variations from normal were found in the following individual cases:

Primary amenorrhea	45,XO
Primary amenorrhea	45,XO/46,XXqi
Short stature, unknown etiology	45,XO/46,XXqi
Cardiomyopathy, unknown etiology	46,XY,13p-
Kern's syndrome	46,XY,15p+

Several cases of primary amenorrhea had normal karyotypes, as did all of the cases of Noonan's syndrome. Our demonstration of normal autosomal and sex chromosomal banding patterns in familial abnormalities such as Noonan's syndrome, pseudohypoparathroidism, and vitamin D-resistant rickets supports the theory that these problems are associated with so-called point mutations rather than small chromosomal rearrangements. Observations of the relationship of dermatoglyphic features to both clinical and chromosomal findings have been continued as part of a long-term project.

Cytogenetic studies on the effect of Diazepam (Valium) on human chromosomes: This study of blood samples from healthy young adults given Valium I.V. for oral surgery has been completed. There was no significant chromosomal damage found following a single large dose. The results of the study, done in collaboration with Dr. E. Driscoll (NIDR-CP), have been submitted for publication.

Karyotypic evaluation of potentially abnormal tissues: Several short-term collaborative projects are in progress. A controlled study of the effects of maternal protein and/or carbohydrate restriction upon the chromosomes of newborn Rhesus monkeys is being done with Dr. J. Sever (NINDS-PR). This project

has so far given negative results and will be completed in several months. Karyotyping of short-term cultures of endothelial tissues from X-irradiated Rhesus monkey brain is partially completed. This project is in collaboration with Dr. Wm. Caveness (NINDS-LEN) and was initiated to determine if chromosomal changes accompany the abnormal endothelial proliferations found at certain intervals after X-ray-induced brain damage. Karyotyping of virally-induced mouse ascites and solid tumors is in progress with Dr. M. Sklar (NIAID-LVM). A strain of mice carrying the translocation T1Wh is being used with mice not carrying a chromosomal marker in tumor transplantation experiments to differentiate between host and transformed donor cells. These observations may help to determine the mechanism of tumor induction by spleen cells treated *in vitro* with certain mouse viruses. This project should be completed in several months.

Publications:

None

Serial No. NIAMDD-LEP-14
1. Experimental Pathology
2. Molecular Pathology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Antigenic and biochemical analysis of biological materials in normal and disease states.

Previous Serial Number: None

Principal Investigator: Jean D. Sipe, Ph.D.

Other Investigators: Thomas F. Ignaczak, M.D.

Cooperating Units: None

Man Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Human Amyloid of Unknown Origin (AUO).

Anti-TAUO antibodies: Ouchterlony titration of previously prepared anti-serum to tissue AUO (TAUO), usually present in patients with "secondary amyloidosis" revealed that antibody activity appeared 6 weeks after initial inoculation with crude denatured TAUO. Efforts to raise antibodies to highly purified TAUO contained in polyacrylamide gel slices and to TAUO covalently coupled to agarose have been unsuccessful. Current efforts to repeat the successful immunization regimen suggest that multiple determinants in the crude TAUO may be important to obtaining precipitating antibodies, as may be the site of inoculation.

Highly purified anti-TAUO immunoglobulin has been obtained by passing anti-TAUO serum over columns of extensively purified TAUO coupled to agarose. Good recovery of antibody activity can be obtained by elution with acetic acid followed by neutralization with Tris. This technique has also been employed to remove inactive antibodies after radiolabeling with ^{125}I, to yield a preparation with nearly 100% antibody activity.

Repeated efforts to isolate the putative serum precursor (SAUO) on anti-TAUO agarose columns have been unsuccessful. Subsequent chromatography of TAUO on both anti-TAUO and control immunoglobulin agarose columns showed a remarkably high affinity of TAUO for nonantibody immunoglobulins. Anti-TAUO agarose has been employed successfuly to purify SAUO and TAUO after ^{125}I-SAUO binding

by anti-TAUO and the concentration dependence of ^{125}I-SAUO binding by anti-TAUO and control immunoglobulin agarose suggests that the antibodies are active after linkage and that perhaps the antigenic determinants on SAUO recognized by the immobilized antibody are not sterically available or that chemical equilibrium does not favor their retention from serum by anti-TAUO agarose.

Radioimmunoassay of SAUO: Because of the high affinity of SAUO and TAUO for immunoglobulins, the standard RIA techniques which depend upon precipitation of immunoglobulins for separation of the antigen-antibody complex cannot be used. However, a competitive RIA for TAUO has been developed using immobilized anti-TAUO. A similar assay for SAUO awaits preparation of antiserum, for the antiserum to the 55 residue TAUO purified in this laboratory appears to be directed largely to determinants which are buried or inaccessible in the higher molecular weight cross-reacting serum protein (SAUO).

High Molecular Weight SAUO: The approximately 180,000 MW SAUO antigenically reactive fraction has been labeled with ^{125}I, and radioactive SAUO can be detected in immunoelectrophoresis with purified anti-TAUO by autoradiography. The biochemical nature of radiolabeled SAUO isolated by precipitation with purified anti-TAUO is now under investigation.

Murine Amyloid Fibril Protein: Murine amyloidosis has previously been found to be an acceptable model for the human secondary disease (J. Histochem. Cytochem. 19: 16, 1971). When column purified spontaneous mouse amyloid (SMA) was coupled to agarose, it was capable of absorbing antibodies from antiserum raised to induced mouse amyloid (IMA). The purified antibodies reacted with a protein of α mobility present in commercial mouse serum and both young and amyloidotic GP serum. There was no reaction with C_3H serum, however. Antibodies to MOPC 511 chains were detected in the antiserum IMA and were not retained by the SMA affinity column. The purified antibodies are now being used in immunofluorescence studies on the pathogenesis of SMA and IMA being carried out in this section.

Immobilized Pyrrolidonecaroxylate Peptidase (PCAase): Kwiatkowska, et al., in this section, have purified a highly stable PCAase from K. cloacae which can be completely coupled to cyanogen bromide activated agarose, when the concentration of enzyme is 100 units/ml. The activity retained after coupling was 30% toward PCA-alanine and 70% toward PCA-paraaminobenzoic acid, respectively. The immobilized enzyme has been used repeatedly in column form without significant loss of activity. Experiments are in progress to use this enzyme to obtain total hydrolysis with high yields of PCA from the amino terminus of such proteins as immunoglobulin heavy and lambda light chains.

Publications:

None

ANNUAL REPORT
LABORATORY OF CHEMICAL BIOLOGY
NIAMDD

The Laboratory of Chemical Biology continues its interests in research activities on the nature of relationships between structure and function in proteins, synthetic and immunologic approaches to the mechanisms of polypeptide chain folding and, more recently, the beginnings of some work on the isolation and study of human interferon. Work on the regulation of protein biosynthesis has now terminated in the laboratory due to the transfer of a research group to another institute.

I. Relationships between protein sequence and three-dimensional structure

A. Studies on the relationship between the amino acid sequences and the three-dimensional structures of proteins: the mechanism of folding of protein chains

Studies are continuing on the mechanisms of folding of polypeptide chains using staphylococcal nuclease and fragments derived from it as models. It is clear from statistical consideration that protein folding must take place through a highly restricted set of pathways since the process in much too rapid, physiologically, to involve a totally random trial and error process. Studies with the formation of native-like analogs of Staph. nuclease through the complementation of overlapping fragments of the protein have suggested that stable three-dimensional structures with nuclease function can be formed in more than one way. Mixing of a fragment containing residues 50-149 of nuclease with a second fragment containing residues 1-126 simultaneously yields, in 1 minute, two alternative ordered complexes, type I and II, resembling nuclease. In type I complex, the segment containing residues 1 to 48 of Nuclease-(1-126) binds to Nuclease-T-(50-149) and residues 49 to 126 of Nuclease-(1-126) protrude from the ordered structure. In type II complex the segment containing residue 1 to some residue between 114 and 123 of Nuclease-(1-126) forms the ordered structure with the segment from some residue between 114 and 123 to 149 of Nuclease-T-(50-149). The unused portions of the polypeptide chains protrude from the ordered structure. After limited digestion of the mixture of Nuclease-(1-126) and Nuclease-T-(50-149) with trypsin in the presence of pdTp and Ca^{2+}, the protruding residues are digested leaving type I and II complexes composed of Nuclease-(1-48) and Nuclease-T-(50-149) and of Nuclease-(1-126) and Nuclease-(111-149), respectively. The ratio of the population of type I complex to type II is approximately 1 to 1 at 2 minutes after mixing of Nuclease-(1-126) and Nuclease-T-(50-149). Either the change of molar ratio of Nuclease-(1-126) to Nuclease-T-(50-149) or the presence of pdTp and Ca^{2+} at the time of mixing of the two fragments appears to have no effect on the population ratio of the two complexes formed. The population ratio of type I to type II complex increased with increase in the incubation time at 6^{o} and reached approximately 2:1 at 70 hours incubation. The results indicate that the difference in the conformational energy between type I and II complexes is approximately 0.4 kcal at 6^{o}.

In order to obtain pure nuclease of sufficient quantity for these experiments, the purification methods of staphylococcal nuclease from culture media

of the organisms were modified. The purification involves ammonium sulfate fractionation followed by gel filtration on Sephadex G75 and affinity chromatography on a Sepharose-pdTp column, using 0.5 M guanidine hydrochloride, pH 7, to elute the enzyme. Recoveries were up to 90% and the resulting material has been crystallized. Preparation of material for crystallization of type II complex is also in progress. The complex formed from Nuclease-(1-126) and Nuclease-(99-149) is digested with trypsin for 10 minutes at 23° in the presence of pdTp and Ca^{2+} to remove residues 99 to 110. Removal of amino-terminal residues of Nuclease-(111-149) and of carboxy-terminal residues of Nuclease-(1-126) will be tried using aminopeptidases and carboxypeptidases, respectively in order to obtain type II complex without the overlapping amino acid sequence. This complex will be used for crystallization.

A major effort is being made to determine with a high degree of certainty that the gross three-dimensional structure of the active nuclease analogs formed by complementation of fragments is very similar to that of the native, unmodified enzyme. This is important because many of the conclusions about the structural and functional aspects of nuclease have come from studies of the fragment complexes. It has been reported earlier that the material known as nuclease-T consisting of residues (6-48) and residues (49-149) is extremely similar to the native nuclease molecule. A detailed three-dimensional structure analysis will shortly be undertaken when equipment is finally installed. The crystallographic work will also deal with the three-dimensional structure of the complex in which the first of the two fragments contains only (6-47). It is then planned to synthesize by the solid phase method of Merrifield analogs of the native sequence to permit an estimate of how much change is permissible in small details of chemistry without loss of the capacity to form a functional protein geometry.

At the present time action is being taken to establish complete peptide synthesis capability. A "rapid phase" peptide synthesis apparatus was designed and is presently being built which is capable of handling the gram amounts of aminoacylated resin necessary to yield sufficient target peptide to provide a usable supply of semisynthetic nuclease T' crystals. The rapid phase synthesis procedure was developed in this laboratory and will be used with planned alterations in the deblocking and coupling steps of the synthesis. The standard Merrifield synthesis performed in suitably modified reaction vessels will be used contingent on the failure of the rapid phase approach to produce an activity generating synthetic nuclease-T(6-48) peptide. t-Boc-ε-TFA lysine (residue 48) resin and t-Boc-Proline (residue 47) resin have been prepared and their degree of substitution determined.

The elucidation of the tertiary structure of semisynthetic nuclease T' would demonstrate for the first time that a largely synthetic enzyme derivative can form a three-dimensional structure closely resembling that of the native protein. Success at this level of investigation will allow for the immediate extension to the synthesis and crystallization of totally synthetic nuclease T'. Improvements in the synthetic method made during the course of this investigation may contribute substantially to the technology designed to produce clinically important synthetic hormones and enzymes. (Taniuchi, Bonhert, Parker, Vigna)

The rapid solid phase synthesis method mentioned above has been under study during the past few months with respect to increasing the efficiency and completeness of coupling of amino acids during successive steps. The introduction of slight modification in coupling times and in the control of the synthesis through careful analysis of end groups after each step has permitted the synthesis of several peptides of 3-16 amino acids in length with encouraging levels of purity. These technological modifications will be of value in the snythetic studies referred to above. (Corley, Curd, Anfinsen)

B. Immunological approaches to the determination of conformational equilibria in staphylococcal nuclease

We have previously shown that the interaction of antibodies with nuclease and fragments of nuclease may be used to quantitate the conformational equilibria (K_{conf}) of polypeptide fragments of nuclease in solution. This has been accomplished by the preparation of antibodies to whole nuclease and to fragments, the sequential immunoabsorption of the antisera to purify antibodies to a specific antigenic region of nuclease or the nuclease fragments, and the measurement of the inhibition of the nuclease-antinuclease reaction by polypeptides. Our objective in the current work has been to develop a general immunologic approach to the determination of the conformational equilibria of proteins using staphylococcal nuclease as a model. The effect of temperature and nuclease ligands, Ca(II) and pdTp, on the K_{conf} values of nuclease were evaluated so as to determine if this conformational parameter is a useful physico-chemical measure of the solution structure of proteins with various perturbants.

As previously described, non-precipitating antibody populations to the sequence 99 to 126 have been purified by sequential immunoabsorption of hyperimmune sera prepared using native nuclease or the nuclease fragments (99-149) and (1-126). Antibodies prepared from goats immunized with whole nuclease so fractionated were called anti-$(99\text{-}126)_N$; antibodies from goats immunized with the fragment (99-149) and so fractionated were called anti-$(99\text{-}126)_R$ and anti-$(127\text{-}149)_R$. The anti-$(99\text{-}126)_R$ antibody populations form soluble complexes with their homologous antigen, are operationally monospecific, but show heterogeneity with regard to binding affinity and isoelectric points on isoelectric focusing. The binding data when analyzed by Scatchard analysis indicate a small subpopulation with a K_a of 1.2×10^7 M^{-1} and a larger population with a K_a of 1×10^9 M^{-1}. Using a simple interpretive model, we have previously estimated the conformational equilibrium of the polypeptide fragment (99-149) using anti-$(99\text{-}126)_N$ (assumed to be specific for the native conformation of the distinct antigenic determinant between 99 and 126). Conversely, we have now measured the conformational equilibrium of native nuclease by employing anti-$(99\text{-}126)_R$ (assumed to be specific for the unfolded conformations of the distinct antigenic determinant between 99 and 126) and a new method of direct inhibition of precipitation. The K_{conf} of nuclease at 25° is 2900 ± 1400; that is, 1 of 2900 molecules of nuclease is totally or partially in an unfolded conformation at any moment. These values were consistent over a wide range of concentrations. The K_{conf} of nuclease at 4 and 39° were 3900 and 400, respectively. In the presence of the ligands Ca(II), or Ca(II) and pdTp, K_{conf} values of 6500 or 3000 to 5000 were estimated, respectively. These results are consistent with

the known stabilization of the native conformation of nuclease by these ligands and are consistent with the explanatory model being used.

The immunologic approach to the study of the conformational equilibria of other proteins will be developed by studying other antigenic determinants on nuclease with other antibodies specific for other regions of the protein. (Furie, Schechter, Anfinsen, Eastlake)

In other current work and attempt is being made to isolate other anti-nuclease antibody populations and study their interaction with nuclease with regard to its conformation as determined from X-ray crystallography and spectroscopy. Solid phase peptide synthesis was employed to prepare peptides from various regions of the amino acid sequence. These peptides have been used to isolate populations of antibodies specific for regions wother than 99-149. These antibodies will be used to study the role of conformational specificity in the interaction of antibodies with nuclease and in the structure-function relationships of nuclease and its derivatives.

In results to date, antibodies eluted with synthetic peptide 53-69 inactivate nuclease. Antibodies eluted with synthetic peptide 43-50, on the other hand, had no effect on activity. (Curd, Schechter, Anfinsen)

C. Nuclear magnetic resonance studies of proteins

Although much has been learned of the solution properties of staphylococcol nuclease, the mechanism of enzymatic acitivity is still unclear. The X-ray diffraction determination of the structure has not given detailed information about the geometry of the active site, the exact orientation of the substrate, or the electronic properties of the residues in the active site. High resolution nuclear magnetic resonance spectroscopy allows inferences about the environments and effective pKa values of groups in the protein. Perturbation of the active site protons by the calcium ion cofactor or substrate analogs may be used to study the effects of these on the protein structure. The paramagnetic lanthanide ions may be used to measure distances in the macromolecule by their effects on proton chemical shift and line widths. It has been the object of this project to study the geometry of the active site of staphylococcal nuclease by these methods.

The perturbation of the four imidazole C2 proton resonances in the 220 MHz NMR spectrum of nuclease by lanthanide ions bound to the metal binding site of nuclease has been examined to determine the distance from the bound metal to the histidine residues in solution. For lanthanide shift ions [Eu(III)] the chemical shift of an observed resonance by a rare earth metal is a function of $1/r^3$, where r is the distance between the observed residue and the metal. Assuming the angular dependence term is unity, the relative distances between each of the four histidines and the metal binding site was found to be within 5% of the relative distances calculated from the crystallographic model. For lanthanide broadening reagents [Gd(III) or Dy(III)], the broadening of the observed resonance by the rare earth metal is a function of $1/r^6$. Using this relationship the relative distances between each of the four histidine resonances determined from the crystallographic model. The measurements of the transverse

and longitudinal relaxation times of the C2 protons was also used to determine the correlation time, τ_c, for nuclease as 2.6×10^{-9} sec. Using this value, the absolute distances between the metal ion and the histidine residues was determined and found to agree within about 10% for three of the four histidine residues (numbers 46, 121 and 124) and less well for residue 8. From these studies it would appear that the solution structure of nuclease is similar to the crystal structure (the reasons for the single discrepant result are unclear) and that the paramagnetic lanthanide ions permit the measurement of intraprotein distances in solution. (Schechter, Furie).

D. Synthetic studies of protein structure and function

The most direct way of studying relationships between structure and function of proteins is by total or partial synthesis of the molecule with the substitution of normally occurring amino acids by other amino acids or amino acid analogs.

Solid phase peptide synthesis has been utilized to introduce amino acids with altered properties into the sequence of the peptide that complexes with the native fragment of ribonuclease, residues 21-124, to produce a semisynthetic complex. The small peptide of 15 residues will form an active complex with 21-124 if the native sequence of 1-15 is used. We have introduced the following variations. At position 12 of the 1-15 peptide we have added 4-fluoro-L-histidine in place of the naturally occurring histidine. We have also added the 1-methyl-L-histidine and 3-methyl-L-histidine derivatives. At position 9 of the sequence we have substituted glycine or leucine for the native glutamic acid. To expand this study, we have undertaken the synthesis of 4-methyl-L-histidine. We have used sulfoethyl Sephadex ion exchange chromatography to effect the purification of the crude synthetic material, followed by Sephadex gel filtration to obtain the purified peptide. The peptides are then characterized by activity assays, inhibition curves, difference spectroscopy, and other probes of structural integrity with respect to their ability to form functional complexes with the native 21-124 fragment. (Dunn, Chaiken, Taylor)

Analogs of semisynthetic ribonuclease-S' have been made for active site and conformationally important residues in the (1-20) fragment. A major effort has been made to prepare ^{13}C-enriched and ^{19}F-containing synthetic fragments which have been used to study the interaction of (1-20) fragment with ribonuclease-S-(21-124) by carbon 13 and fluorine 19 NMR spectroscopy. It has been shown that such selective labelling can be achieved synthetically and that the products provide valuable tools for measuring structural parameters for specific residues in going from disordered (denatured) to ordered (native) environments. The techniques involved also have indicated the application to the study of the mechanism of action of ribonuclease-A and -S. Ongoing carbon 13 enrichment studies with oxytocin have helped to define residues of this peptide which interact directly with neurophysin. (Chaiken, Taylor)

In related studies on the interaction between polypeptides to yield functional complexes, studies have been continued on the characterization of the products formed by mixing in solution neurophysin to oxytocin and vasopressin. This convenient interacting system may serve as a good model for the formation of similar complexes involving other hormones, such as ACTH.

Isolation of major neurophysins at high purity has been achieved from bovine posterior pituitaries. Binding of ^{14}C-labelled oxytocin and vasopressin to neurophysin I and II has been studied by equilibrium dialysis. A neurophysin assay method has been developed based on this dialysis procedure. The pure neurophysins have been analyzed by ultracentrifugation for properties of aggregation. This has been carried out for neurophysins I and II alone and in the presence of oxytocin and a tripeptide analogue. The stability of the neurophysins has been examined with respect to its ability to withstand disulfide interchange, again alone or with tripeptide ligand. Antibody preparation is being attempted by development of protein carrier species for administration to rabbits. (Chaiken, Curd, Taylor, Randolph, Fischer, Sanchez)

II. Projects on the structure and function of several protein systems

A. Regulation of histidine biosynthesis in Salmonella typhimurium

Studies are continuing on the elucidation of the mechanism by which expression of the histidine operon is regulated in response to the needs of the microorganism under a variety of conditions. Previous work suggested that this system is autogenously regulated -- that is, expression of the genes of the operon is regulated by a protein specified by one of those very genes. The protein in question is known to also function as an enzyme, the so-called G enzyme that catalyzes the first step in the pathway for histidine biosynthesis. A role for this enzyme as a regulatory protein that controls expression of the histidine operon is indicated by the findings of several different projects.

Strains have been isolated in which a single mutation in the structureal gene for the G enzyme leads not only to synthesis of structurally damaged G enzyme but also to loss of regulation of the entire histidine operon. The G enzyme has been shown to interact with high affinity and high specificity with aminoacylated histidyl-tRNA. Such properties fulfill an important requirement for any regulatory protein that may influence expression of the histidine operon The enzyme does not, however, differentiate between normal (wild-type) histidyl-tRNA and immature (hisT) histidyl-tRNA. This finding eliminates the possibility that the difference in the regulatory properties in the two species of tRNA is mediated by G enzyme recognition and suggests that the difference is mediated by the interaction of a G enzyme-tRNA complex with the DNA of the histidine o operon. The G enzyme has been found to interact specifically with the DNA of the histidine operon carried in the genome of a defective Ø80 transducing bacteriophage. Furthermore, competition experiments, using various preparations of radioactively labeled and unlabeled DNA suggest that the enzyme interacts with the regulatory region of the operon. A purified in vitro system was developed for transcribing the histidine operon carried in the genome of a defective Ø80 transducing bacteriophage. Messenger-RNA specific for the histidine operon was found to be synthesized in this system from the correct ("sense") strand of the phage DNA. This mRNA had a sedimentation constant identical with that of histidine operon mRNA synthesized in vivo. Addition of purified G enzyme to the in vitro system caused a severe and specific inhibition of transcription of the histidine operon, demonstrating that the enzyme functions as a negative control element for the histidine operon in vitro and suggesting that it plays a similar role in vivo. Studies are under way to develop an in vitro system for synthesizing the enzymes of the histidine operon from the DNA of a defective Ø80

transducing phage that carries the operon in its genome. Because the in vitro system involves both E. coli and S. typhimurium, it is complicated by the problem of the bacterial restriction-modification systems. For this reason, a good deal of effort has gone into obtaining "restrictionless" strains and into other possible ways to solve the restriction problem. (Goldberger, Meyers, Mullinix, Kronenberg, Kovach, Levinthal, Bruni)

B. Isolation, structural determination, and synthesis of human int[illegible]eron

Both leucocyte and fibroblast interferons are removed from crude tissue fluids by columns of anti-leucocyte interferons attached to Sepharose-4B. The antibody was prepared in sheep, using, as antigen, material that had been partially purified by gel filtration through Sephadex-G100 columns. Many of the impurities in the crude fibroblast interferon were presumably not recognized by the sheep antibodies induced by leucocyte interferon. Fibroblast interferon was, therefore, much more effectively purified as the result of this "common denominator" approach. Both varieties of interferon exhibit molecular weights of approximately 20,000-25,000 although binding to higher molecular weight protein occurs. Since the amounts of interferon that we are likely to obtain, even with unusually good recovery, will be very small, we have resorted to an approach involving radioactivity. Human fibroblast tissue cultures, induced with poly I - poly C have been incubated in the presence of highly radioactive amino acids. Any given tissue culture is exposed to only two or three amino acids containing radioactivity. Following purification by affinity chromatography, gel filtration, and disc gel electrophoresis, the minute amounts of radioactive interferon are placed in an automatic Edman sequencing apparatus. At any step of degradation in which radioactive counts are removed from the protein chain being degradated, the phenylthiohydantoin may be identified by thin layer chromatography. These studies are in the early stage, but the principle should hopefully be applicable to the determination of at least the first 20 or 30 amino acids in the interferon polypeptide backbone. Other work is in progress to establish whether or not the carbohydrate side chains of interferon may be removed without loss of anti-viral activity. (Anfinsen, Bose, Corley, Gurari-Rotman)

C. The structure and function of the phenylalanine-tRNA and histidine-tRNA syntheses from Salmonella typhimurium

The aminoacyl-tRNA synthetases are enzymes which catalyze the first step in protein biosynthesis by attaching amino acids to their cognate tRNA chains. The molecular basis for this specificity is still very much in the dark and its elucidation by means of NMR-spectroscopy, various fluorescence techniques, equilibrium dialysis and eventally X-ray crystallography constitute the major objective of this project.

In the final step of the purification by means of affinity chromatography the enzyme was either retarded or completely retained by the resin depending on the degree of phenylalanine substitution on the agarose. The homogeneity of the enzyme was demonstrated by analytical ultracentrifugation and disc gel electrophoresis. A molecular weight of 210,000 has been established by means of equilibrium centrifugation and gel chromatography. In preliminary SDS electrophoresis experiments two bands corresponding to molecular weights of

53,000 and 100,000 have been found. It seems likely that the enzyme consists of four identical subunits, but additional experiments will be required for the clarification of the subunit structure. Several amino acid analyses of the enzyme have been performed.

The major future focus will be on the tRNA enzyme interactions whereby mainly fluorescence techniques and NMR-spectroscopy will be employed. It is hoped that aside from the overall association constant a more detailed picture of this highly specific interaction will eventually emerge from these studies. NMR studies should reveal possible hydrogen bonds formed between the tRNA molecule and the enzyme and also allow the detection of a possible conformational change of this ligand due to the binding. It is hoped that from such studies general inferences can be made about the interaction of proteins with nucleic aicds. (Schiller, Schechter, Eastlake)

D. An immunochemical study of hemoglobin and its derivatives

It has been shown in this laboratory that the interaction of antibodies with nuclease and fragments from this protein may be used to quantitate the conformational equilibria of polypeptide chains and proteins in solution. This has been accomplished by the preparation of antibodies to whole nuclease and fragments, the purification of non-precipitating antibodies to single antigenic regions in the carboxy-terminal end of the protein by sequential affinity chromatography and the quantitation of the interaction of these antibodies with antigens, singly and in various combinations. It is the purpose of the present work to extend these studies and analyses to the hemoglobin A (Hgb A) molecule in its various states of oxidation, liganding and with mutations. We will try to produce antibodies specific for the conformation of various hemoglobins with mutations, such as sickle cell hemoglobin (Hgb S), and to use these antibodies to detect, quantitate, and study the conformation of the abnormal hemoglobins under various conditions.

Relatively large amounts of antibodies to the oxy- and ferri- states of Hgb A and Hgb S have been purified to homogeneity with respect to their antiseric specificities by ammonium sulfate and immunoadsorption chromatography. The reaction of these antibodies to their homologous, and some heterologous, antigens has been studied by the quantitative precipitin reaction. The quantitative precipitin reaction of each antibody preparation to its antigen with various ligands (H_2O, O_2, CO, DPG) has been measured and some significant differences have been found. The functional significance of these differences is not yet certain.

Our antibody work may lead to new methods to detect and quantitate abnormal hemoglobin forms. Of equal importance, it may lead to ways of detecting conformational properties of hemoglobin molecules which can not be detected by other means (such as differences between oxy- and ferri- hemoglobins) and which may have significance for the function of hemoglobin. The methods may also be useful for studying changes in the conformation of the deoxy-Hgb S, before the cooperative gellation, which may be useful in a rigorous description of the gel process. (Schechter, Furie, Curd, Eastlake)

E. Isolation and characterization of myoglobin from paramecium

A chromatographic fraction isolated from lysed paramacia was shown to contain 5 heme proteins on standard polyacrylamide disc gels. In SDS, however, only one heme protein path is seen, suggesting that the 5 paths are electrophoretic variants with the same molecular weight. Preliminary studies on determining the molecular weight of these species has resulted in average molecular weights of 14,000 - significantly less than the vertebrate myoglobins studied.

Many invertebrate hemoglobins have been identified in a variety of sources. This is the first instance of a molecule of this class appearing in the protozoa. Of special interest is the possible role of this molecule which may be quite different than the role ascribed to these pigments in higher organisms as well as the apparent structural difference where the heme group is covalently bound to the protein. (Steers)

III. Quantitative affinity chromatography

Studies were initiated to develop techniques for utilizing affinity chromatography as a quantitative tool for studying the interactions between macromolecules and specific ligands. Chromatography of highly purified staphylococcal nuclease on columns of deoxythymidine-5'-phosphate-3'-(p-aminophenyl)phosphate-Sepharose has been carried out in the presence of various concentrations of the competing active site ligand, deoxythymidine-d',5'-diphosphate. The amount of affinity matrix has been varied by dilution with unsubstituted Sepharose 4B. Equations to describe the resulting behavior were derived from first principles.

The free soluble ligand, thymidine 3',5'-diphosphate is able to effectively compete with the immobilized ligand and thus interrupt the affinity process. This results in elution of the enzyme from the column at a position in the eluant that changes inversely with the soluble ligand concentration. This parameter varies directly in the amount of affinity matrix present and directly on the magnitude of the bonding constant of the competing ligand to the protein. This technique, when coupled with equations we have derived, allows the determination of binding constants for soluble ligand to protein as well as insoluble ligand to protein. Furthermore, given one affinity matrix, a series of inhibitions could be evaluated with respect to the strength of binding to a given protein. (Dunn, Chaiken)

Serial No. NIAMDD-LCB-1
1. Lab. of Chemical Biology
2. Protein Conformation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the relationship between the amino acid sequence and the three-dimensional structures of proteins: the mechanism of folding of protein chains

Previous Serial Number: NIAMDD-LCB-1

Principal Investigators: Hiroshi Taniuchi
Janice L. Bohnert
Diana S. Parker

Man Years:

Total: 2.5
Professional: 0.9
Other: 1.6

Project Description:

Objectives: To find the mechanism of folding of a protein on the basis of the amino acid sequence

Methods Employed and Major Findings: As reported in the previous year a model of folding of staphylococcal nuclease has been developed. It is assumed that under physiological conditions two similar native conformations of nuclease exist at higher and lower energy states - an activated and a ground state - and that the folding of disordered nuclease into the activated state occurs statistically with little change in the conformational energy. The transition from the activated to the ground state would occur by development of the unique state of cooperative interactions throughout the three dimensional structure. In order to test this hypothesis the relationship between the binding of ligands to Nuclease-T' (see the previous reports) and the stabilization of the structure was studied.

The rate of unfolding of Nuclease-T' at pH 8, 20° was determined as a function of concentration of the ligands deoxythymidine-3'5'-diphosphate (pdTp) and Ca^{2+} on the basis of the rate of exchange between free fragment, Nuclease-T-(50-149) and labeled fragment, Nuclease-T-(50-149) incorporated in the structure of Nuclease-T'. Sepharose-nuclease, in which staphylococcal nuclease was covalently bound to Sepharose (agarose) by the cyanogen bromide activation method, was used to remove pdTp prior to separation of Nuclease-(50-149) and Nuclease-T' by affinity chromatography with Sepharose-pdTp (see the previous reports for other details of the methods). The rate constant of unfolding of unliganded Nuclease-T' was 4.6×10^{-4} S^{-1}. Those of Nuclease-T' bound with pdTp, with Ca^{2+} and with both pdTp and Ca^{2+} were 9.0×10^{-5}, 1.6×10^{-4} and 2.2×10^{-5} S^{-1},

respectively. The association constants of pdTp and Ca^{2+} with Nuclease-T' were found to be $1.0x10^4$ and $2.0x10^2$ M^{-1}, respectively. Those of pdTp and Ca^{2+} with Nuclease-T' plus Ca^{2+} and with Nuclease-T' plus pdTp were $4x10^5$ and $1.4x10^4$ M^{-1}, respectively. The results indicate that the magnitude of free energy change involved in the binding of either ligand to Nuclease-T' is increased by approximately 2 kcal when the other ligand is bound. Similarly, the coupling of the local interactions, between the ligands and the segments of the polypeptide chains at the binding site, with the interaction system operating throughout the three-dimensional structure results in the stabilization of the structure of Nuclease-T' without a large change of conformation.

The interaction of Nuclease-T-(50-149) and Nuclease-(1-126) simultaneously yield in 1 min two alternative ordered complexes, type I and II, resembling nuclease (see the previous reports). In type I complex, the segment containing residues 1 to 48 of Nuclease-(1-126) binds to Nuclease-T-(50-149) and residues 49 to 126 of Nuclease-(1-126) protrude from the ordered structure. In type II complex the segment containing residue 1 to some residue between 114 and 123 of Nuclease-(1-126) forms the ordered structure with the segment from some residue between 114 and 123 to 149 of Nuclease-T-(50-149). The unused portions of the polypeptide chains protrude from the ordered structure. After limited digestion of the mixture of Nuclease-(1-126) and Nuclease-T-(50-149) with trypsin in the presence of pdTp and Ca^{2+}, the protruding residues are digested leaving type I and II complexes composed of Nuclease-(1-48) and Nuclease-T-(50-149) and of Nuclease-(1-126) and Nuclease-(111-149), respectively. Type I complex is assumed to be more stable against thermal denaturation than type II complex. Therefore, the distribution of type I and II complexes at the equilibrium state in the mixture of Nuclease-(1-126) and Nuclease-T-(50-149) should be consistent with their thermodynamic stabilities. However, if the energy state differentiating the two types of complexes develops only after Nuclease-(1-126) and Nuclease-T-(50-149) chains refold into the two specific spatial arrangements resembling type I and II complexes, the populations of the two ordered complexes formed from the two fragments in 1 min will be determined by a statistical mechanism independent of the difference in their conformational energy. This hypothesis was tested by determining the populations of the two ordered complexes formed from the two fragments as a function of time at 6°.

The mixture of the two fragments was digested with trypsin in the presence of the ligands to remove the redundant segments after incubation for given times at 6°. The four component fragments of the two complexes were quantitatively separated by gel filtration on a Sephadex G50 column with 30% acetic acid and ion exchange chromatography on a phosphocellulose column at 55°. Trypsin was completely removed by affinity chromatography on a Sepharose-soybean trypsin inhibitor column and gel filtration on a Sephadex G75 column before the separation of the four fragments. The quantity of the separated fragments was determined by amino acid analysis. The ratio of the population of type I complex to type II is approximately 1 to 1 at 2 min after mixing of Nuclease-(1-126) and Nuclease-T-(50-149). Either the change of molar ratio of Nuclease-(1-126) to Nuclease-T-(50-149) or the presence of pdTp and Ca^{2+} at the time of mixing of the two fragments appears to have no effect on the population ratio of the two complexes formed. The population ratio of type I to type II complex increased with increase in the incubation time at 6° and reached approximately 2:1 at 70 hours incubation. The results are consistent with the

above hypothesis and indicate that the difference in the conformational energy between type I and II complexes is approximately 0.4 kcal at 6°.

In order to obtain pure nuclease of sufficient quantity for these experiments, the purification methods of staphylococcal nuclease from culture media of the organisms were modified. Crude nuclease eluted with 50% saturated ammonium sulfate from phosphocellulose which was used to absorb the enzyme in the culture medium (see the previous reports of LCB) was brought to 90% saturation with $(NH_4)_2SO_4$ at pH 6. The precipitate was discarded. The supernatant solution was brought to 100% saturation with $(NH_4)_2SO_4$ at pH 8. The precipitate was collected and purified by gel filtration on a Sephadex G75 column. The final purification was achieved by affinity chromatography on a Sepharose-pdTp column (see the previous report of LCB) using 0.5 M guanidine HCl, pH 7 to elute the enzyme. The modified methods allow recovery of up to 90% of the nuclease present in the crude material. The nuclease purified by the present method has been crystallized.

Preparation of material for crystallization of type II complex is in progress. The complex formed from Nuclease-(1-126) and Nuclease-(99-149) is digested with trypsin for 10 min at 23° in the presence of pdTp and Ca^{2+} to remove residues 99 to 110. Removal of amino-terminal residues of Nuclease-(111-149) and of carboxy-terminal residues of Nuclease-(1-126) will be tried using aminopeptidases and carboxypeptidases, respectively in order to obtain type II complex without the overlapping amino acid sequence. This complex will be used for crystallization.

Significance to Biomedical Research: The structure and function of biological macromolecules are regulated and maintained by weak non-bonded interactions. The system of complementation of nuclease fragments developed here offers an excellent test object for direct examination of the principles underlying the non-bonded interactions involved in the formation of three-dimensional structure. The system can also be used to study the mechanism of catalytic activity of the enzyme.

The difference in conformational energy of 0.4 kcal between type I and II complexes must be expressed as a small difference in some of the atomic coordinates. Therefore, if the atomic coordinates of the two ordered complexes are accurately determined, the system may afford a testing object for current theories used to calculate the conformational energy of proteins. These tested theories, if successful, will aid in the prediction of the three-dimensional structure of a protein on the basis of the amino acid sequence.

Proposed Course of Project:

1) Determination of the atomic coordinates of type I complex (Nuclease-T') by X-ray diffraction study.

2) Crystallization and determination of the atomic coordinates of type II complex by X-ray diffraction study.

3) Calculation of the conformational energy of type I and II complexes on the basis of the atomic coordinates.

4) Study of the kinetics and thermodynamics involving the interactions of complementing fragments of nuclease, ribonuclease A, cytochrome c and other pertinent proteins.

5) Study the mechanism of formation of the "correct" disulfide bonds in proteins (ribonuclease, lysozyme, chymotyrpsinogen, etc.) after reduction and reoxidation.

6) Study the principles involved in the formation of three-dimensional structure and the regulation of function of larger proteins.

Publications:

Light, A., Taniuchi, H., and Chen, R.F.: A kinetic study of the complementation of fragments of staphylococcal nuclease. J. Biol. Chem. 249, 2285, 1974.

Serial No. NIAMDD-LCB-2
1. Lab. of Chemical Biology
2. Protein Conformation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the relationship between the primary and tertiary structure of protein: the crystallization and X-ray diffraction study of semisynthetic nuclease T'.

Previous Serial Number: NIAMD-LCB-1

Principal Investigators: Robert A. Vigna
Hiroshi Taniuchi

Cooperating Unit: Christian B. Anfinsen
(NIAMDD, LCB, Protein Chemistry)

Man Years:

Total: 0.9
Professional: 0.8
Other: 0.1

Project Description:

Objective: To crystallize and define by X-ray diffraction methods the three-dimensional structure of semisynthetic nuclease T'.

Rationale: The background for this work, based mainly on observations published by this laboratory, is presented to establish the timeliness of the objective.

Staphylococcal nuclease, the 149 residue extracellular endonuclease of Staphylococcus aureus (Foggi strain), has been extensively investigated. The primary and tertiary structures of this enzyme are known and the solution properties have been thoroughly studied. The physico-chemical properties of nuclease compare favorably with the structural information obtained by X-ray diffraction techniques.

Nuclease T is an enzymically active complementing system formed by the non-covalent association of the peptide nuclease-T-(6-48) with either the peptide nuclease-T-(49-149) or nuclease-T-(50-149) of nuclease. Nuclease T is a product of the protection afforded nuclease by the ligands 3',5'-deoxythymidine diphosphate (pdTp) and calcium ion (Ca^{++}) to tryptic digestion. The enzymic activity of nuclease T toward both DNA and RNA substrates is 8-10% that of native nuclease. The solution conformation of nuclease and nuclease T are very similar as judged by a number of criteria. However, the heat transitions measured for nuclease and nuclease T indicate a reduced stability for nuclease T compared to nuclease. Tritium-hydrogen exchange experiments suggest enhanced solvent accessibility for nuclease T compared to nuclease. Reconstituted nuclease T (nuclease T') is identical to nuclease T by all established standards.

The nuclease T complementing system has been utilized effectively as a test object for evaluating the structural and functional relationships of nuclease. Previous reports from this section have dealt with the use of the nuclease T complex to obtain folding and unfolding rate information and to calculate the activation free energy of the unfolding process as a function of the liganded state of the enzyme. A systematic series of studies were initiated as a result of the successful solid phase peptide synthesis of the fragment nuclease-T-(6-47). The enzymically active complex formed by complementation of synthetic nuclease-T-(6-47) and nuclease-T-(49-149) is known as semisynthetic nuclease T'. The role of specific amino acids in catalysis and conformation stabilization have been examined indirectly by Merrifield synthesis of suitably substituted semisynthetic analogues of nuclease T'. The high resolution X-ray structure of liganded nuclease determined elsewhere provided a defined system against which the results of these studies could be compared.

At this time, the inherent failing of the Merrifield solid phase peptide synthesis routine is the production of target peptide in poor yield. Decreased synthetic yields present a difficult peptide purification problem especially for synthetic fragments as large as nuclease-T-(6-48). The purification of synthetic nuclease-T-(6-47) was greatly simplified by a scheme which takes advantage of the resistance of liganded nuclease T' to digestion by trypsin. Semisynthetic nuclease T' recovered by this procedure is in highly purified form and possibly capable of crystallizing without further purification.

Recently, crystals of nuclease T and nuclease T' consisting of the peptides nuclease-T-(6-48) and nuclease-T-(49-149) have been prepared. These crystals have been shown to be isomorphous with and in the same space group as native nuclease. The current X-ray analysis of nuclease T' in conjunction with the information obtainable by the fragment exchange experiments described in the previous report support the immediate extension of this work to crystalline semisynthetic nuclease T'.

Methods Employed and Major Findings: The early stages of this work have dealt primarily with the purification of nuclease, preparation of nuclease T and the isolation of all the component peptide fragments of the nuclease T complex. A recent modification developed in this section for the purification of nuclease from phosphocellulose cakes gave nuclease preparations of approximately 40% purity after two ammonium sulfate fractionations and G-75 gel filtration chromatography (see the other report of the Section on Protein Conformation). Nuclease recovered at this stage of the purification procedure was in sufficiently pure form to conveniently prepare nuclease T.

Nuclease T has been prepared and purified using the established methods (see previous reports for details). Nuclease is digested in the presence of pdTp and Ca^{++} with trypsin at 25α and pH 8.0 for 2 to 4 hours. The resulting nuclease T is then purified on a phosphocellulose column developed with an aommonium acetate buffer gradient. The preparation of all the component fragments of nuclease T has been accomplished by the chromatographic techniques routinely used in this laboratory. Nuclease-T-(6-48) is isolated from nuclease-T-(49-149) and nuclease-T-(50-149) on a G-50 Sephadex column eluted with 30% acetic acid. Nuclease-T-(49-149) and nuclease-T-(50-149) are purified on a column of phosphocellulose developed with an ammonium acetate buffer

gradient. Purity of all the components has been established by two-dimensional peptide mapping. The overall strategy of this project dictates the need for ample quantities of purified nuclease T component peptides.

Experiments are presently underway designed to isolate, characterize and crystallize the nuclease T' complex formed by the complementation of nuclease-T-(6-47) and nuclease-T-(49-149). Synthetic nuclease-T-(6-47) is the peptide commonly prepared for semisynthetic nuclease T' studies. The nuclease-T-(6-47) peptide is synthesized in preference to the parent nuclease-T-(6-48) since the loss of lysine residue 48 has no apparent effect on the activity or structure of nuclease T. Synthesis of nuclease-T-(6-47) also eliminates one coupling step over synthesis of nuclease-T-(6-48) thereby providing a slight improvement in yield of the target peptide. All the solution studies of semisynthetic nuclease T' and its analogue complexes have been performed with the synthetic [nuclease-T-(6-47) + nuclease-T-(49-149)] complementing system. Crystallization and X-ray examination of the natively obtained [nuclease-T-(6-47) + nuclease-T-(49-149)] complex to establish its crystallographic identity with the [nuclease-T-(6-48)] + [nuclease-T-(49-149)] complex is fundamental to previous semisynthetic nuclease T' investigations.

Parent nuclease-T-(6-48) was incubated at 25α for 5 hours at pH 8.0 with a 1:200 enzyme to substrate ratio of carboxypeptidase B. Preliminary analyses suggest that COOH-terminal lysine residue removal proceeds in a yield of at least 70%. The carboxypeptidase B treated peptide was chromatographed on a column G-50 Sephadex. The material eluting in the volume identical unmodified nuclease-T-(6-48) was complemented with an equal molar amount of nuclease-T-(50-149). After tryptic digestion of this mixture in the presence of pdTp and Ca^{++}), the resulting complex was isolated on a column of phosphocellulose. An enzymically active major fraction eluting before the expected position of the [nuclease-T-(6-48) + nuclease-T-(50-149)] form of nuclease T was recovered. The low yield of complex obtained after the tryptic digestion step above will necessitate the repeat of this experiment to obtain sufficient modified complex from which the carboxypeptidase B produced nuclease-T-(6-47) can be isolated and characterized. Characterization will involve comparative peptide mapping using residue specific stains, amino acid analysis and time course digestion with the COOH-terminal exopeptidase carboxypeptidase C. The nuclease-T-(6-47) will be complemented with nuclease-T-(49-149) isolated and crystallized. Crystallization will be attempted using the same conditions established for nuclease T': [nuclease-T-(6-48) + nuclease-T-(49-149)]. The resulting crystalline complex will be subjected to preliminary X-ray diffraction analysis to establish the highly isomorphous relationship of this species to nuclease T and nuclease T'.

At the present time action is being taken to establish complete peptide synthesis capability for the Protein Conformation section. A "rapid phase" peptide synthesis apparatus was designed and is presently being built which is capable of handling the gram amounts of aminoacylated resin necessary to yield sufficient target peptide to provide a usable supply of semisynthetic nuclease T' crystals. The rapid phase synthesis procedure was developed in this laboratory and will be used with planned alterations in the deblocking and coupling steps of the synthesis. The standard Merrifield synthesis performed in suitably modified reaction vessels will be substituted upon the failure of

the rapid phase approach to produce an activity generating synthetic nuclease-T-(6-48) peptide. t-Boc-ε-TFA lysine (residue 48) resin and t-Boc-Proline (residue 47) resin have been prepared and their degree of substitution determined.

Significance to Biomedical Research: The elucidation of the tertiary structure of semisynthetic nuclease T' would demonstrate for the first time that a largely synthetic enzyme derivative can form a three-dimensional structure closely resembling that of the native protein. Success at this level of investigation will allow for the immediate extension to the synthesis and crystallization of totally synthetic nuclease T'. Improvements in the synthetic method made during the course of this investigation may contribute substantially to the technology designed to produce clinically important synthetic hormones and enzymes.

At the level of protein structure, observations described in previous reports from this section suggest that the nonbonded interactions which form and stabilize the native structure of a protein operate cooperatively throughout the three-dimensional structure. Individual interactions however are not equivalent in their contribution toward stabilization of the native conformation of a protein. The X-ray crystallographic investigation of semisynthetic nuclease T' and suitably modified analogues is essential to the study and understanding of nonbonded interactions. The crystallographic approach supplemented with the thermodynamic information obtainable through labelled fragment exchange experiments for these semisynthetic analogues (see previous report) should prove to be an effective combination of methods for studying nonbonded interactions of proteins. The information obtained from these studies will contribute to a more precise definition of the nature and role of nonbonded interactions which are essential for maintaining the integrity of the biological system.

Proposed Course of Research:

1) Crystallization and X-ray diffraction study of semisynthetic nuclease T' containing synthetic nuclease-T-(6-48) and native nuclease-T-(49-149).

2) Crystallization and X-ray diffraction study of semisynthetic nuclease T' analogues to investigate at the level of atomic resolution the folding interactions and catalytic mechanism of nuclease T.

3) Kinetic and thermodynamic studies involving the interaction of synthetically modified nuclease-T-(6-48) fragments with native nuclease-T-(49-149).

4) Crystallization and characterization of nuclease T' complexes composed of natural fragments truncated by carboxypeptidase C or aminopeptidase M digestion to determine the minimal amino acid sequence requirement for production of an enzymically active crystalline complex. Such a study is designed to reduce the number of operations required for the total synthesis of nuclease T'.

5) Total synthesis, crystallization and X-ray diffraction study of nuclease T'.

Serial No. NIAMDD-LCB-3
1. Lab. Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Functional and physicochemical characterization of neurophysin-neurohypophyseal hormone complexes

Previous Serial Number: NIAMDD-LCB-2

Principal Investigators: Irwin M. Chaiken
Raymond E. Randolph
Hope C. Taylor
Ernst Fischer

Other Investigators: John Curd
Guillermo R. Sánchez

Man Years:

Total: 2.1
Professional: 1.35

Project Description:

1) To isolate and purify neurophysins from the posterior pituitary glands of selected mammalian species.
2) To study the characterics of binding of the neurophysins to the neurohypophyseal hormones, oxytocin and vasopressin.
3) To characterize the aggregation and stability properties of neuro-neurophysin, both in the presence and in the absence of ligands.
4) To prepare antibodies directed against the neurophysins, for the study of a possible neurophysin precursor and for conformational analysis of the neurophysins as isolated.

Methods Employed: Isolation of major neurophysins at high purity has been achieved from bovine posterior pituitaries. Binding of ^{14}C-labelled ligand to neurophysin I and II has been studied by equilibrium dialysis. A neurophysin assay method has been developed based on this dialysis procedure.

The pure neurophysins have been analyzed by ultracentrifugation for properties of aggregation. This has been carried out for neurophysins I and II alone and in the presence of oxytocin and a tripeptide analogue.

The stability of the neurophysins has been examined with respect to its ability to withstand disulfide interchange, again alone or with tripeptide ligand. Antibody preparation is being attempted by development of protein carrier species for administration to rabbits.

Major Findings: Equilibrium dialysis experiments have shown that methionyl-tyrosyl-[^{14}C]phenylalanine amide bonds to each of neurophysins I and II with 1:1 stoichiometry but with binding data exhibiting some complexities at low ligand concentrations.

Ligands have a significant effect on the aggregation properties of the neurophysins. Preliminary studies suggest that ligands also have an effect on protein stability.

Antibody preparation is still in progress.

Significance to Biomedical Research: The studies undertaken should allow an increased understanding of the at present ill-defined biological function of the neurophysins. The information hopefully will help to describe the biosynthetic origin and fate of the neurohypophyseal hormones before entering the pituitary and after release. The protein-hormone complex also serves as a model of hormone-receptor interaction.

Proposed Course of Action:

1) Continuance of studies on the effect of ligands binding on equilibrium properties of the neurophysins and extension to kinetic properties.

2) Definition of chemical detail of the binding function of the neurophysin.

3) Continuance of studies with neurophysin antibodies.

Serial No. NIAMDD-LCB-4

1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of protein conformation and biological function with semisynthetic noncovalent peptide-protein complexes.

Previous Serial Number: NIAMDD-LCB-2

Principal Investigator: Irwin M. Chaiken

Other Investigator: Hope C. Taylor

Cooperating Units: 1) Laboratory of Chemistry, National Heart and Lung Institute, NIH (Edward A. Sokolosky).
2) National Institute of Child Health and Development, NIH (Jack S. Cohen).
3) Polymer-Crystal Physics Laboratory, National Bureau of Standards, Gaithersburg, Md. (James R. Lyerla, Jr.)

Man Years:

Total: .4
Professional: .3
Other: .1

Project Description:

1) To prepare, by chemical synthesis, polypeptides and protein fragments which interact with corresponding native proteins or protein fragments to form biologically active complexes.

2) To obtain purified synthetic products suitable for detailed study as part of semisynthetic peptide-protein complexes.

3) To use the semisynthetic complexes for studying the conformational and functional properties of the particular systems in question, notably by studies of synthetic analogues containing carbon 13 enrichment and fluorine 19 label at specific amino acid loci.

Methods Employed: Several systems have been investigated, including bovine pancreatic ribonuclease-S' and the neurophysin-neurohypophyseal peptide hormone complexes. In the former case, a specific protein fragment, ribonuclease-S-(1-20) has been studied with respect to the noncovalent complex formed upon addition to the complementary fragment ribonuclease-S-(21-124). Semisynthetic analogues of the native complex have been obtained by chemical synthesis of ribonuclease-S-(1-20) variants. With the latter, the microenvironments of specifically altered loci have been examined by spectroscopic methods.

For the interacting systems of the neurophysins with the peptide hormones oxytocin and vasopressin, synthesis of oxytocin and vasopressin derivatives also is being carried out.

Major Findings: Analogues of semisynthetic ribonuclease-S' have been made for active site and conformationally important residues in the (1-20) fragment. A major effort has been made to prepare ^{13}C-enriched and ^{19}F-containing synthetic fragments which have been used to study the interaction of (1-20) fragment with ribonuclease-S-(21-124) by carbon 13 and fluorine 19 NMR spectroscopy. It has been shown that such selective labelling can be achieved synthetically and that the products provide valuable tools for measuring structural parameters for specific residues in going from disordered (denatured) to ordered (native) environments. The techniques involved also have indicated the application to the study of the mechanism of action of ribonuclease-A and -S.

Ongoing carbon 13 enrichment studies with oxytocin have helped to define residues of this peptide which interact directly with neurophysin.

Significance to Biomedical Research: The above studies allow the description of structural bases for conformation and biological function for the specific cases of study are applicable to an understanding of other proteins as well. Thus, these studies contribute to the goal of understanding the underlying principles of normal and abnormal protein function.

Proposed Course of Action:

1) Continuance of project for semisynthetic ribonuclease-S, with specific attention to important synthetic analogues for which functional and conformational information will lead to an increased understanding for these proteins in particular and for other proteins in general.

2) Study of neurophysin-peptide hormone systems, with further attention to synthetic introduction of spectroscopic probes into residues of the peptide hormone which are involved in the binding function with the neurophysins.

Publication:

Chaiken, I.M.: Carbon 13 as a probe of helix formation in semisynthetic ribonuclease-S', J. Biol. Chem. 249, 1247, 1974.

Serial No. NIAMDD-LCB-5
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Semisynthetic analogues of Ribonuclease S'.

Previous Serial Number: NIAMDD-LCB-3
NIAMDD-LCB-2

Principal Investigator: Ben M. Dunn

Other Investigators: Irwin M. Chaiken
Hope Taylor

Cooperating Units: Physical Sciences Laboratory, Laboratory of Chemical Physics, NIAMDD

Man Years:

Total: .85
Professional: 0.7
Other: .15

Project Description:

Objectives:

1) To examine the relationship between functional group character and catalytic activity in a model protein.

2) To develop methods for functional purification of semisynthetic derivatives.

3) To examine the influence of side chain character on stability of tertiary structure and peptide-protein interactions.

Methods Employed: Solid phase peptide synthesis has been utilized to introduce amino acids with altered properties into the sequence of the peptide that complexes with the native fragment of ribonuclease, residues 21-124, to produce a semisynthetic complex. The small peptide of 15 residues will form an active complex with 21-124 if the native sequence of 1-15 is used. We have introduced the following variations. At position 12 of the 1-15 peptide we have added 4-fluoro-L-Histidine in place of the naturally occurring histidine. We have also added the 1-methyl-L-histidine and 3-methyl-L-histidine derivatives. At position 9 of the sequence we have substituted glycine or leucine for the native glutamic acid. To expand this study, we have undertaken the synthesis of 4-methyl-L-histidine.

We have used sulfoethyl Sephadex ion exchange chromatography to effect the purification of the crude synthetic material, followed by Sephadex gel filtration to obtain the purified peptide. The peptides are then characterized

by activity assays, inhibition curves, difference spectroscopy, and other probes of structural integrity with respect to their ability to form functional complexes with the native 21-124 fragment.

Major Findings: By mixing the crude solid phase derived 1-15 peptides with native 21-124 a complex is formed between synthetic peptides of proper sequence and the 21-124 fragment. Sulfoethyl sephadex chromatography under controlled conditions of pH and ionic strength is then sufficient to separate the stable complex from the synthetic peptide of incorrect sequence. Additional steps of gel filtration yield the synthetic peptide isolated from the 21-124 native fragment as confirmed by amino acid composition.

The methylated derivatives, 1-methyl-histidine 12-synthetic(1-15) and 3-methyl-histidine 13-synthetic(1-15) are without activity in the crude state. We have obtained some evidence from affinity chromatography for binding of the 3-methyl derivative to the 21-124 fragment. After purification utilizing the ion-exchange procedure we have developed the 3-Me-His derivative had 10% of the activity of the native complex and the 1-Me-His derivative was totally inactive.

The peptides with alterations in the side chain at position 9 have been shown to yield catalytically active complexes when mixed with native 21-124. This property has been exploited to achieve purification of the crude material resulting in production of purified 1-15 peptides with side chain modification in position 9. Binding constants have been determined by activity titration curves. In addition, we have demonstrated that these analogue peptides will prevent the inactivation of RNase-S-(21-124) by the disulfide-interchange-enzyme. These studies will aid in elucidating the crucial question of the organization of polypeptides into functional tertiary structure.

The [4-fluoro-L-Histidine 12]-synthetic(1-15) peptide, which we have previously shown to bind to RNase-S-(21-124) but which lacks activity, has been further characterized by examination of the titration curves of the histidine residues of the semisynthetic complex via high resolution proton nuclear magnetic resonance spectroscopy. We have observed pKa values of 6.4 for His 119 and 6.8 for His 105 of the complex.

Significance to Biomedical Research: A deeper understanding of the factors that govern catalytic activity and the formation of tertiary structure is indispensible to the eventual understanding of the molecular basis of disease. The design of improved or corrected proteins must be based on an understanding of structure-function relationships in protein chemistry.

Proposed Course of Project:

1) Further characterization of the peptides already in hand with respect to interactions with the 21-124 fragment.

2) Design, synthesis, purification, and characterization of other derivatives in this series.

3) Correlation of functional properties with side chain characteristics.

Serial No. NIAMDD-LCB-6
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Kinetic studies of the mechanism of action of staphylococcal nuclease.

Previous Serial No.: NIAMDD-LCB-4

Principal Investigator: Ben M. Dunn

Man Years:

Total: 0.2
Professional: 0.2

Project Description:

Objectives:
1) To quantitate the steady-state kinetic parameters for hydrolysis of deoxythymidine 5'-p-nitrophenylphosphate 3'-phosphate by staphylococcal nuclease as a function of pH.
2) To characterize the mechanism of reaction of Woodward's Reagent K with staphylococcal nuclease.

Methods Employed: Hydrolysis of Woodward's reagent K has been examined by UV, nuclear magnetic resonance, and infrared spectroscopy. The intermediate and products have been identified by assignment of the resulting absorption curves. The kinetic process has been quantitated with respect to pH variation and mechanism utilizing a circulating pH stat system developed in this laboratory. The reaction with the protein has been quantitated by observing loss of activity with respect to pH, reagent concentration and active site ligands.

Major Findings: The reaction of Woodward's reagent K has been shown to involve the intermediacy of a ketohetenimine which reacts rapidly with either water or carboxyl groups of staphylococca nuclease. Kinetic parameters have been derived by curve fitting to a mathematical model incorporating all features of the mechanism. Protection against modification was observed for active site ligands.

Significance to Biomedical Research: Understanding the principles of catalysis by macromolecules will allow a deeper understanding into fundamental life processes. Clearer appreciation of the relationship between structure and function will enable rational decisions to be made concerning correction of defects in molecular diseases.

Proposed Course of Project:

1) Continued probing into the catalytic events of staph. nuclease reactions using rapid reaction techniques.

2) Further characterization of the reaction products and the modified protein.

Publication:

Dunn, B.M., DiBello, C. and Anfinsen, C.B.: The pH-dependence of the steady-state kinetic parameters for staphylococcal nuclease catalyzed hydrolysis of deoxythymidine-3'-phosphate-5'-p-nitrophenylphosphate in H_2O and D_2O. J. Biol. Chem. 248, 4769, 1973.

Dunn, B.M., Shrager, R.I., Anfinsen, C.B.: Kinetics of hydrolysis of Woodward's reagent K and reaction with staphylococcal nuclease, J. Biol. Chem., in press.

Serial No. NIAMDD-LCB-7
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Quantitative affinity chromatography

Previous Serial No.: none

Principal Investigator: Ben M. Dunn

Other Investigator: Irwin M. Chaiken

Man Years:

Total: 0.3
Professional: 0.3

Project Description:

Objectives:
1) To develop a procedure for the utilization of affinity chromatography to measure binding constants.
2) To obtain insights into the process of affinity chromatography of macromolecules.

Methods Employed: Chromatography of highly purified staphylococcal nuclease on columns of deoxythymidine-5'-phosphate-3'-(p-aminophenyl)phosphate-Sepharose has been carried out in the presence of various concentrations of the competing active site ligand, deoxythymidine-3',5'-diphosphate. The amount of affinity matrix has been varied by dilution with unsubstituted Sepharose 4B. Equations to describe the resulting behavior were derived from first principles.

Major Findings: The free soluble ligand, thymidine 3',5'-diphosphate is able to effectively compete with the immobilized ligand and thus interrupt the affinity process. This results in elution of the enzyme from the column at a position in the eluant that changes inversely with the soluble ligand concentration. This parameter varies directly in the amount of affinity matrix present and directly on the magnitude of the binding constant of the competing ligand to the protein. This technique, when coupled with equations we have derived, allows the determination of binding constants for soluble ligand to protein as well as insoluble ligand to protein. Furthermore, given one affinity matrix, a series of inhibitions could be evaluated with respect to the strength of binding to a given protein.

Significance to Biomedical Research: This procedure will allow the rapid evaluation of binding ability for a compound to a particular protein. This could be of great utility in screening programs that seek inhibition for a specific metabolic pathway or in programs that wish to quantitate binding of

hormones to proteins where an activity assay is difficult or inexact. It should be of utility in examining the interaction of derivatized peptides with complementary protein fragments.

Proposed Course of Project:

1) Further quantitation of binding constants in the staphylococcal nuclease system.

2) Extension of the procedure to other systems under study in this lab.

Serial No. NIAMDD-LCB-8
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Isolation, structural determination, and synthesis of human interferon

Previous Serial Number: NIAMDD-LCB-11

Co-investigators: Christian B. Anfinsen
Sikta Bose
Lila Corley
Dalia Gurari-Rotman

Man Years:
Total: 3.5
Professional: 2.5
Other: 1

Project Description:

Objectives: The availability of fairly large amounts of crude human interferon from NIAID, through a commercial contract involving the use of human leucocyte cell cultures challenged with Newcastle virus, or human fibroblasts challenged with poly I - poly C, makes it feasible to consider the isolation of pure agent for subsequent structural and biological study. Eventual synthesis of human interferon would appear to be the only practical approach to the use of the substance clinically since it is present in such minute amounts in biological sources.

Methods Employed and Major Findings: Both leucocyte and fibroblast interferons are removed from crude tissue culture fluids by columns of anti-leucocyte interferon attached to Sepharose-4B. The antibody was prepared in sheep using, as antigen, material that had been partially purified by gel filtration through Sephadex-G100 columns. Many of the impurities in the crude fibroblast interferon were presumably not recognized by the sheep antibodies induced by leucocyte interferon. Fibroblast interferon was, therefore, much more effectively purified as the result of this "common denominator" approach. The fibroblast product was bound with what was interpreted to be a markedly lower affinity for the antibodies produced during the early stages of immunization. However, it could be harvested efficiently from the crude starting material when a carrier protein (bovine serum albumin and, later, cytochrome c) was added to the eluting buffers to counteract losses, presumably due to adsorption on purification and assay equipment. Both varieties of interferon exhibit molecular weights of approximately 20,000-25,000 although binding to higher molecular weight proteins occurs.

Since the amounts of interferon that we are likely to obtain, even with unusually good recovery, will be very small, we have resorted to an approach involving radioactivity. Human fibroblast tissue cultures, induced with poly I - poly C, have been incubated in the presence of highly radioactive amino acids. Any given tissue culture is exposed to only two or three amino acids containing radioactivity. Following purification by affinity chromatography, gel filtrations, and disc gel electrophoresis, the minute amounts of radioactive interferon are placed in an automatic Edman sequencing apparatus. At any step of degradation in which radioactive counts are removed from the protein chain being degradated, the phenylthiohydantoin may be identified by thin layer chromatography. These studies are in the early stage, but the principle should hopefully be applicable to the determination of at least the first 20 or 30 amino acids in the interferon polypeptide backbone.

Other work is in progress to establish whether or not the carbohydrate side chains of interferon may be removed without loss of anti-viral activity.

Significance to Biomedical Research: As mentioned above, knowledge about this broad spectrum antiviral substance will hopefully permit the organic synthesis of the molecule, or of the active part of its structure, for clinical testing.

Serial No. NIAMDD-LCB-9
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Improvements in the solid-phase synthesis of polypeptides

Previous Serial Number: NIAMDD-LCB-10

Principal Investigators: Lila Corley
John Curd
Christian B. Anfinsen

Man Years:

Total: 0.3
Professional: 0.2
Other: 0.1

Project Description:

Objectives: The times usually devoted to the required steps in the Merrifield solid-phase synthetic scheme do not permit the manual addition of more than about two or three amino acid residues per day. This means that considerable time is needed even for the synthesis of rather short polypeptides, as we have found in our previous syntheses of nuclease fragments. We have therefore set out to modify the synthetic scheme in order to increase the speed of synthesis and to minimize those losses attributable to the long reaction times under harsh conditions which are presently used in this method.

Methods Employed: Methods were developed for following the coupling and deblocking reactions during solid-phase synthesis in small funnels so that the time course could be determined. An apparatus was constructed permitting the rapid addition of reagents with mixing by bubbling rather than by rocking. The process of shrinking and swelling the resin in order to increase washing efficiency was studied and employed.

Major Findings: Since the report of June 30, 1973, the rapid solid phase method has been used to synthesize several peptides of 3 to 16 amino acids in length. The modified naphthaldehyde method has been employed to study the coupling after each amino acid addition. This is correlated with amino acid analysis.

Significance to Biomedical Research: Our studies suggest that the rapid method of solid phase synthesis is as effective as standard Merrifield procedures while allowing substantial savings of time necessary for synthesis.

Proposed Course of Project: Further modifications of the protecting groups employed and the methods for purification of products will be developed.

Serial No. NIAMDD-LCB-10
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Isolation and characterization of myoglobin from paramecium.

Previous Serial No.: NIAMDD-LCB-13

Principal Investigator: Edward Steers, Jr.

Man Years:

Total: 1.5
Professional: 1
Other: 0.5

Project Description:

Objectives: To purify the heme containing a protein in Paramecium and characterize it fully including sequential analysis.

Methods: Paramecia are grown as previously described in five gallon carboys on grass extract and Aerobacter cloacae (Steers, E., Jr., PNAS 48:867, 1962). The cells are harvested by passage through a commercial cream separator. Final concentration is achieved by centrifugation in a clinical centrifuge. The packed cells are resuspended in approximately two volumes of Tris-HCl buffer, pH 7.5 which is adjusted to 0.2 M with raffinose to prevent the swelling and lysis of mitochondria. The suspension of cells is then homogenized in a Dounce tissue homogenizer at 0°. The lysed cells are then centrifuged retaining the supernatant solution. This solution, which appears pink in color is further fractionated with ammonium sulfate retaining the 50 to 65% fraction. Following dialysis the same is chromatographed on columns of Sephadex G-75 which is monitored spectrophotometrically at 416 nm. The appropriate fractions are pooled and concentrated by precipitating with ammonium sulfate at 65% saturation.

The precipitate is dialyzed against Tris buffer, pH 7.5 and electrophoresed on 7 1/2% polyacrylamide gels after which the fine heme containing bands are cut out of the gels and the protein eluted in Tris-HCl buffer.

Major Findings: The chromatographic fraction isolated as described above shows five heme-containing bands when electrophoresed on standard polyacrylamide disc gels. When electrophoresed on disc gels containing 0.1% SDS, only one heme-containing zone appears. This suggests that the individual bands are electrophoretic variants which are the same molecular weight. It has been possible to cut each band (numbered 1 thru 5) from the disc gels and elute the protein by incubation, the gel slices in Tris-HCl buffer, pH 7.5. The eluted

proteins appear 100% pure and reelectrophorese true to form indicating that there is no equilibrium between the electrophoretic variants. Preliminary studies on determining the molecular weight of these species has resulted in average molecular weights of 14,000 - significantly less than the vertebrate myoglobins studied.

Previously reported results also show this myoglobin to differ from the characterized vertebrate forms by apparently having the heme covalently bound or bound more tightly when treated by those procedures which remove the heme from vertebrate myoglobins. The five electrophoretic variants are present in both in oxy and reduced forms showing only a difference in absolute mobility on disc gels. The basis for the existence of five variant forms is unknown at this time.

Of additional interest is the physiologic role of this molecule in Paramecium. It would appear neither to function as a transport substance for oxygen as oxygen freely diffuses through the cell nor does it appear to serve as a storage mechanism being in too small a quantity to serve the normal metabolic needs of the organism under anaerobic conditions.

Significance to Biomedical Research: Many invertebrate hemoglobins have been identified in a variety of sources. This is the first instance of a molecule of this class appearing in the protozoa. Of especial interest is the possible role of this molecule which may be quite different than the role ascribed to these pigments in higher organisms as well as the apparent structural difference where the heme group is covalently bound to the protein. While the spectral and functional characteristics of the molecule are clearly those of the hemoglobin class of compounds, the covalent attachment of the heme is characteristic of the cytochromes.

Proposed Course of Project: Following the successful purification of Paramecium hemoglobin, a full characterization is anticipated including sequence analysis.

Serial No. NIAMDD-LCB-11
1. Lab. of Chemical Biology
2. Protein Chemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Isolation and characterization of trichocysts from *Paramecium aurelia*.

Previous Serial No.: NIAMDD-LCB-12

Principal Investigator: Edward Steers, Jr.

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

This project is terminated.

Serial No. NIAMDD-LCB-12
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Nuclear magnetic resonance studies of the structure and function of staphylococcal nuclease.

Previous Serial Number: NIAMDD-LCB-6 and 7

Principal Investigators: Alan N. Schechter
Bruce Furie

Cooperating Units: Division of Computer Research and Technology (Richard Feldmann); Laboratory of Chemistry, NHLI (Edward Sokoloski)

Man Years:

Total: 0.6
Professional: 0.6

Project Description:

Objectives: Although much has been learned of the solution properties of staphylococcal nuclease, the mechanism of enzymatic activity is still unclear. The X-ray diffraction determination of the structure has not given detailed information about the geometry of the active site, the exact orientation of the substrate, or the electronic properties of the residues in the active site. High resolution nuclear magnetic resonance spectroscopy allows inferences about the environments and effective pKa values of groups in the protein. The paramagnetic lanthanide ions may be used to measure distances in the macromolecule by their effects on proton chemical shift and line widths. It is the object of this project to study the geometry of the active site of staphylococcal nuclease by these methods.

Methods Employed: Continuous wave NMR spectra have been recorded in the Varian HR-220 MHz spectrometer and changes in chemical shift and line width of histidine ring protons evaluated as a function of added metal ion concentration.

The relaxation rate enhancements by paramagnetic metal ions of nuclei of nucleotides bound to nuclease were measured on the Varian HR-220 MHz spectrometer or the Varian XL-100 Spectrometer employing Fourier transform analysis. Measurements were made of the transverse relaxation rates of protons and the longitudinal and transverse relaxation rates of phosphorus nuclei. Internuclear distances between Gd(III) bound to the metal-binding site of nuclease and atoms

of the nucleotide were determined from these data by using the Solomon-Bloembergen equation. These internuclear distances were used with a computer program and graphics display to solve for metal:nucleotide geometries which were consistent with the experimental data. Computer facilities, including the DEC PDP-10 and Adage AGT-30 graphics display computer, were employed.

Major Results: The perturbation of the four imidazole C2 proton resonances in the 220 MHz NMR spectrum of nuclease by lanthanide ions bound to the metal binding site of nuclease has been examined to determine the distance from the bound metal to the histidine residues in solution. For lanthanide shift ions [Eu(III)] the chemical shift of an observed resonance by a rare earth metal is a function of $1/r^3$, where r is the distance between the observed residue and the metal. Assuming the angular dependence term is unity, the relative distances between each of the four histidines and the metal binding site was found to be within 5% of the relative distances calculated from the crystallographic model. For lanthanide broadening reagents [Gd(III) or Dy(III)], the broadening of the observed resonance by the rare earth metal is a function of $1/r^6$. Using this relationship the relative distances between each of the four histidine resonances was also in close agreement with the values determined from the crystallographic model. The measurements of the transverse and longitudinal relaxation times of the C2 protons was also used to determine the correlation time, τ_c, for nuclease as 2.6×10^{-9} sec. Using this value, the absolute distances between the metal ion and the histidine residues was determined and found to agree within about 10% for three of the four histidine residues (numbers 46, 121 and 124) and less well for residue 8. From these studies, it would appear that the solution structure of nuclease is similar to the crystal structure (the reasons for the single discrepant result are unclear) and that the paramagnetic lanthanide ions permit the measurement of intraprotein distances in solution.

We have extended these analyses to the study of the geometry of the bound substrate analog. Previous results from this laboratory have shown that lanthanide ions bind tightly in the metal-binding site of nuclease, inhibit Ca(II)-dependent catalysis, and facilitate the formation of a ternary complex of nuclease:lanthanide metal:nucleotide. Control experiments have shown that the broadening of resonances is due to the effect of the single bound paramagnetic ion and is due neither to substrate analog-enzyme binding or analog-metal complex formation alone. Thus the changes in transverse and longitudinal relaxation times are due to paramagnetic metal related processes and the amount of broadening as a function of metal concentration can be related to the metal-nuclei distance by the Solomon-Bloembergen equation, using the ascertained value of τ_c.

The following results (in Å) were obtained (1st number is NMR result, in parentheses is X-ray determined value): C6-H, 7.8±.3 (7.7); C4'-H, 7.7±.5 (8.4); C5',5''-H, 6.5±.3 (6.5 and 6.7); CH_3, 9.6±.3 (7.1, 7.4 and 8.4); C3',5' P, 6.2±.1 (4.5 and 10). We interpret the agreement as substantial but believe that the discrepancy may indicate a significant difference in the orientation of the substrate in the protein.

Possible solution structures of these bound nucleotides were determined using a computer analysis to solve for metal:nucleotide geometries which were consistent with the NMR data. For 3'5' TDP, a geometry similar to the structure

of the metal:nucleotide complex bound to nuclease determined by X-ray analysis, but with a significant rotation of the base with respect to the ribose ring, was one of the solutions to this computer modeling process. A 12 minute 16 mm motion picture has been produced from the Adage AGT-30 graphics display unit to illustrate the approach, results, and steric significance of this work to understanding the geometry of the nuclease active site.

This approach is currently being extended to analysis of the conformation of 3'-TMP, 5'-TMP, NO_2-phenyl-pdTp, NO_2-phenyl-pdTp-NO_2phenyl, and NO_2-phenyl pdT bound to nuclease. The latter three nucleotides are nuclease substrates; the former are inhibitors. In general, the interatomic Gd(III)-nucleotide nuclei distances correspond closely to those determined by previous crystallographic analysis of the nuclease:thymidine diphosphate complex, but we are interested in the possibility of small, but significant differences from the X-ray. The computer program is being refined so as to allow insertion of the calculated metal-nucleotide structures into the protein within the constraints of Van der Waals contact distances.

Work on the NMR of other proteins has been deferred except for the preparation for publication of previous results.

Significance to Biomedical Research: The high resolution NMR studies on histidine residues give information about the effective pKa values, dielectric constant, and geometry of the active site of nuclease. The study of the effects of lanthanide ions on these histidine resonances gives information confirming the similarity of the crystal and solution studies and the validity of the approach using paramagnetic ions. The detailed geometrical inferences that may be made from understanding the effects of lanthanide ions on proteons (and other nuclei) on the substrate analogue should allow a visualization of the active site in solution with extreme precision. This information will effectively complement that of X-ray diffraction in understanding the structure and mechanism of this enzyme.

These methods should be applicable to other metal binding proteins, especially the biologically important calcium containing proteins, where studies have been hindered by the lack of useful electronic or magnetic properties of calcium ions.

Proposed Course of Project: The collection and analysis of data on the paramagnetic relaxation of nuclei in true substrates and the refinement of computer programs for converting such results into possible three-dimensional structures will be continued.

These and other NMR techniques will be used in our on-going studies of protein structure and function.

Publications:

Cohen, J.S., Fisher, W.R., and Schechter, A.N.: Spectroscopic studies on the conformation of cytochrome c and apocytochrome c. J. Biol. Chem. 249, 1113-1118, 1974.

Furie, B., Griffin, J.H., Feldmann, R.J., Sokolowski, E.A., and Schechter, A.N.: The active site of staphylococcal nuclease: paramagnetic relaxation of bound nucleotide inhibitor nuclei by lanthanide ions. Proc. Natl. Acad. Sci., in press.

Serial No. NIAMDD-LCB-13
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunological properties of staphylococcal nuclease: determination of conformational equilibria.

Previous Serial Number: NIAMDD-LCB-5

Principal Investigators: Bruce Furie
Alan N. Schechter
Christian B. Anfinsen
Ann Eastlake

Cooperating Units: Immunology Branch, NCI (David H. Sachs)

Man Years:

Total: 0.8
Professional: 0.7
Other: 0.1

Project Description:

Objectives: We have previously shown that the interaction of antibodies with nuclease and fragments of nuclease may be used to quantitate the conformational equilibria (K_{conf}) of polypeptide fragments of nuclease in solution. This has been accomplished by the preparation of antibodies to whole nuclease, the sequential immunoabsorption of the antisera to purify antibodies to a specific antigenic region of nuclease, and the measurement of the inhibition of the nuclease-antinuclease reaction by polypeptides. Our objective in the current work has been to develop a general immunologic approach to the determination of the conformational equilibria of proteins using staphylococcal nuclease as a model. The effect of temperature and nuclease ligands, Ca(II) and pdTp, on the K_{conf} values of nuclease were evaluated so as to determine if this conformational parameter is a useful physico-chemical measure of the solution structure of proteins with various perturbants.

Methods Employed: Polypeptide fragments from nuclease have been prepared and purified and antibodies to these and native nuclease have been produced in goats. The antisera have been purified by selective immunoadsorption on Sepharose columns to which proteolytic fragments of nuclease have been covalently bound. By these means antibodies to single antigenic determinants have been prepared which are immuno-specific and non-precipitating.

Carbamylation, primarily of α-NH_2 groups, by $KN^{14}CO$ has been used to label nuclease and its fragments so that direct equilibrium and kinetic measurements

of the interaction with antibody could be made. The labeled polypeptides behave similarly to the unlabelled ones. A double precipitation radioimmunoassay using rabbit anti-goat gamma globulin to precipitate antibody-bound [^{14}C]-polypeptide has been developed for this purpose.

Major Results: As previously described, non-precipitating antibody populations to the sequence 99 to 126 have been purified by sequential immunoabsorption of hyperimmune sera prepared using native nuclease or the nuclease fragments (99-149) and (1-126).

Antibodies prepared from goats immunized with whole nuclease so fractionated were called anti-$(99\text{-}126)_N$; antibodies from goats immunized with the fragment (99-149) and so fractionated were called anti-$(99\text{-}126)_R$ and anti-$(127\text{-}149)_R$. The anti-$(99\text{-}126)_R$ antibody populations form soluble complexes with their homologous antigen, are operationally monospecific, but show heterogeneity with regard to binding affinity and isoelectric points on isoelectric focusing. The binding data when analyzed by Scatchard analysis indicate a small subpopulation with a K_a of $1.2\text{x}10^7$ M^{-1} and a larger subpopulation with a K_a of $1\text{x}10^5$ M^{-1}.

Using a simple interpretive model, we have previously estimated the conformational equilibrium of the polypeptide fragment (99-149) using anti-$(99\text{-}126)_N$ (assumed to be specific for the native conformation of the distinct antigenic determinant between 99 and 126). Conversely, we have now measured the conformational equilibrium of native nuclease by employing anti-$(99\text{-}126)_R$ (assumed to be specific for the unfolded conformations of the distinct antigenic determinant between 99 and 126) and a new method of direct inhibition of precipitation. The K_{conf} of nuclease at 25° is 2900±1400; that is, 1 of 2900 molecules of nuclease is totally or partially in an unfolded conformation at any moment. These values were consistent over a wide range of concentrations. The K_{conf} of nuclease at 4° and 39° were 3900 and 400, respectively. In the presence of the ligands Ca(II), or Ca(II) and pdTp, K_{conf} values of 6500 and 30000 to 50000 were estimated, respectively. These results are consistent with the known stabilization of the native conformation of nuclease by these ligands and are consistent with the explanatory model being used. The new methods used to obtain K_{conf} for nuclease also obviate certain problems in the methods used earlier.

Further studies have been done on the nature of the anti-$(99\text{-}126)_N$ antibody subpopulation which we have extensively used in previous studies. New methods for the preparation of Fab fragments of goat antibodies have been developed. The Scatchard plots of the binding data of these antibodies to nuclease, however, shows the same concavity, with a sharp break at 0.5 antigens per Fab unit. These results indicate that there are indeed two populations of antibodies - with respect to affinity - in this preparation (presumably on a genetic basis) and not negative cooperativity between protein antigen binding sites. Isoelectric focusing studies show most of the protein in two triplets of bands suggesting that this functional approach to antibody purification has resulted in a population which may have only two genetic forms (with post-translational modification accounting for the heterogeneity within each triplet). We are pursuing this work in an attempt to achieve further restriction of heterogeneity.

Significance to Biomedical Research: Conformation plays a major role in the biologic properties of proteins and polypeptide regions. Therefore, one needs means of assessing the conformational properties of proteins. Because such fragments may contain only a very small percentage of the cooresponding native conformation (or, conversely, native proteins contain only a small percentage of unfolded polypeptide), there are often no physical methods capable of such assessment. The immunologic analysis of a K_{conf} therefore provides a parameter of conformation unobtainable by physical methods alone. The method described herein represents a general approach for the determination of K_{conf} of any polypeptide or protein. Requirements include a labelled polypeptide chain, a nonprecipitating antibody which distinguishes between the random and native conformation, and an absolute or relative association constant for the interaction of antibody and its corresponding antigen. The technique may therfore prove useful in elucidating the structure and dynamics of more complicated protein systems, such as those with multiple subunits, lipoproteins, and membrane proteins.

Proposed Course of Project: The immunologic approach to the study of the conformational equilibria of other proteins will be developed by studying other antigenic determinants on nuclease with antibodies specific for other regions of the protein (see also NIAMDD-LCB-14). In addition, we have prepared antibodies to several forms of hemoglobin A and hemoglobin S with the intent of evaluating the effect of ligands on the interaction of conformationally specific anti-hemoglobin antibodies with hemoglobin and hemoglobin fragments (see NIAMDD-LCB-15).

Publications:

Furie, B., Schechter, A.N., Sachs, D.H., and Anfinsen, C.B.: Antibodies to an unfolded form of a helix-rich region of staphylococcal nuclease. Biochemistry 13, 1561-1566, 1974.

Eastlake, A., Sachs, D.H., Schechter, A.N., and Anfinsen, C.B.: Binding of univalent antibody fragments to a distinct antigenic determinant of staphylococcal nuclease. Biochemistry 13, 1567-1571, 1974.

Serial No. NIAMDD-LCB-14
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunological properties of staphylococcal nuclease: isolation and fractionation of antibodies with synthetic polypeptides.

Previous Serial Number: None

Principal Investigators: John Curd
Alan Schechter
Christian B. Anfinsen

Cooperating Units: Immunology Branch, NCI (David H. Sachs)

Man Years:

Total: 0.7
Professional: 0.7

Project Description:

Objectives: Previous investigators in this laboratory have fractionated goat antibodies produced against staphylococcal nuclease. After fractionation two non-precipitating populations were obtained which were operationally specific for antigenic determinants in the amino acid sequences 99-126 and 127-149. These antibodies inactivate nuclease and the k_{on}, k_{off}, and K_{assoc} of these interactions have been determined. A model for conformational equilibria of polypeptides in solution has been proposed and empirical conformational constants have been measured.

The current work is an attempt to isolate other antinuclease antibody populations and study their interaction with nuclease with regard to its conformation as determined from X-ray crystallography and spectroscopy. Solid phase peptide synthesis was employed to prepare peptides from various regions of the amino acid sequence. These peptides have been used to isolate populations of antibodies specific for regions other than 99-149. These antibodies will be used to study the role of conformational specificity in the interaction of antibodies with nuclease and in the structure-function relationships of nuclease and its derivatives.

Methods Employed: Goat antisera to nuclease were produced by repeated immunization in complete Freund's adjuvant. Polypeptides corresponding to amino acid sequences 23-31, 31-39, 43-50, 58-66, and 53-69 were synthesized by standard or modified solid phase techniques. Sepharose-peptide, Aminoethyl-Sepharose-peptide, and Sepharose-nuclease columns were prepared.

Major Results: Several Sepharose-peptide immunoadsorbents were used to fractionate antibodies, which could be eluted from the adsorbent with urea. These populations were not monospecific for the amino acid sequences involved, however, because precipitin formed when the antibodies were combined with nuclease. Specific non-precipitating antibodies have been successfully prepared by eluting antibodies from Sepharose-nuclease with solutions of a synthetic peptide in buffered saline. The antibodies and the peptide were then separated by gel filtration. The antibodies obtained interact specifically with nuclease. Antibodies eluted with peptide 53-69 inactivate nuclease. Antibodies eluted by peptide 43-50 do not inactivate nuclease.

Significance to Biomedical Research: The antibody populations obtained by elution with synthetic peptides allow the investigator to study areas of nuclease which can not be studied when peptide fragments from cyanogen bromide cleavage or limited trypsin digestion are employed. Theoretically the investigator can now pick the sequence or structure to be studied, such as alpha helices, beta bends, and other localized sequences. The isolation of antibody populations may lead to a better understanding of the immunology of nuclease. Antigenic determinants will be localized and compared with the X-ray crystallographic model. Correlations between known or measureable secondary structure and antibody specificity will lead to a better physical understanding of the role of conformation in nuclease-antibody interactions. The technique of eluting specific antibody with synthetic peptides could be generally applicable to the study of the immunological properties of proteins where the primary sequence is known.

Proposed Course of the Project: The properties of the antibody populations described and their interactions with nuclease and its derivatives will be studied. Other regions will be screened for specific antibody.

Serial No. NIAMDD-LCB-15
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: An immunochemical study of hemoglobin and its derivatives

Previous Serial No.: None

Principal Investigators: Alan Schechter
Bruce Furie
John Curd
Ann Eastlake

Cooperating Units: Clinical Hematology Brance, NIAMDD (Neal Young)

Man Years:

Total: 1.2
Professional: 0.8
Other: 0.4

Project Description:

Objectives: It has been shown in this laboratory that the interaction of antibodies with nuclease and fragments from this protein may be used to quantitate the conformational equilibria of polypeptide chains and proteins in solution. This has been accomplished by the preparation of antibodies to whole nuclease and fragments, the purification of non-precipitating antibodies to single antigenic regions in the carboxy-terminal end of the protein by sequential affinity chromatography and the quantitation of the interaction of these antibodies with antigens, singly and in various combinations.

It is the purpose of the present work to extend these studies and analyses to the hemoglobin A (Hgb A) molecule in its various states of oxidation, liganding and with mutations. Specifically, we are attempting to detect conformational differences among ferro-, ferri-, oxy-, and carboxy-hemoglobins and to study the effect of diphosphoglyceric acid (DPG) on hemoglobin conformation. We will try to produce antibodies specific for the conformations of various hemoglobins with mutations, such as sickle cell hemoglobin (Hgb 5), and to use these antibodies to detect, quantitate, and study the conformation of the abnormal hemoglobins under various conditions.

Methods Employed: Hemoglobin A has been chromatographically purified from washed red blood cells. Hgb S has also been purified chromatographically, in large amounts, from blood of individuals heterozygous for the sickle gene or from individuals with sickle cell disease. The α and β chains from both Hgb A and S have been chromatographically separated, after p-chloromercuribenzoate treatment of the native molecule, and polypeptide fragments from these isolated

chains have been prepared by proteolytic digestion (chemical and enzymatic) and chromatographic separation. Solid phase synthesis of some peptides has been initiated.

Hgb A and Hgb S, in the ferri- and oxy- conformations have been used as immunogens in goats to raise antibodies. When high anti-Hgb titers have been obtained, large amounts of antibody have been purified by ammonium sulfate precipitation and immunoadsorption chromatography on Sepharose columns to which the various hemoglobin species have been bound.

Methods for eluting these specific antibodies have been developed and the physico-chemical characterization of their interaction with the hemoglobin molecules and their derivatives has been initiated. So far the quantitative precipitin method has been used most extensively in these studies, but work on other methods including measurements of oxygen affinity and difference spectroscopy has begun.

Major Results: Large amounts of antibodies to the oxy- and ferri-states of Hgb A and Hgb S have been purified with respect to their hemoglobin specificity by ammonium sulfate and immunoadsorption chromatography. The reaction of these antibodies to their homologous, and some heterologous, antigens has been studied by the quantitative precipitin reaction. The quantitative precipitin reaction of each antibody preparation to its antigen with various ligands (H_2O, O_2, CO, DPG) has been measured and some significant differences have been found. The functional significance of these differences is not yet certain.

Attempts to obtain soluble antibody-hemoglobin complexes (mono-specific antibody preparations) by sequential immunoadsorption and elution from Sepharose-hemoglobin columns have been unsuccessful so far.

In addition we have attempted to purify antibodies specific for the conformations of mutant hemoglobins by immunoadsorption chromatography but we still have significant cross-reactivity in our preparations.

Significance to Biomedical Research: We have shown the antibody method to be a valid measure of conformational properties of proteins and polypeptides not ascertainable by other means. Further, antibodies have wide utility for detection and quantitation of proteins in biological fluids. The physicological and pathological importance of hemoglobin, and its mutant species, is well appreciated. Our antibody work may lead to new methods to detect and quantitate abnormal hemoglobin forms. Of equal importance, it may lead to ways of detecting conformational properties of hemoglobin molecules which can not be detected by other means (such as differences between oxy- and ferri- hemoglobins) and which may have significance for the function of hemoglobin. The methods may also be useful for studying changes in the conformation of the deoxy-Hgb S, before the cooperative formation, which may be useful in a rigorous description of the gel process.

Proposed Course of Project: The studies outlined here will be continued in an attempt to reach the goals noted above.

Serial No. NIAMDD-LCB-16
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1 1973 through June 30, 1974

Project Title: The structure and function of the phenylalanine-tRNA and histidine-tRNA syntheses from Salmonella typhimurium.

Previous Serial Number: NIAMDD-LCB-8

Principal Investigators: Peter Schiller
Alan N. Schechter
Ann Eastlake

Cooperating Unit: Istituto di Chimica Biologica, Universitá di Napoli, Naples, Italy (Prof. Francesco De Lorenzo, Dr. Paola Di Natale)

Man Years:

Total: 1.8
Professional: 1.3
Other: 0.5

Project Description:

Objectives: The aminoacyl-tRNA synthetases are enzymes which catalyze the first step in protein biosynthesis by attaching amino acids to their cognate tRNA chains. These enzymes must catalyze these reactions with a high degree of fidelity in order to prevent altered proteins from occurring. Any particular aminoacyl-tRNA synthetase must therefore recognize and interact with its cognate tRNA and amino acid in a highly specific fashion. The molecular basis for this specificity is still very much in the dark and its elucidation by means of NMR-spectroscopy, various fluorescence techniques, equilibrium dialysis and eventually X-ray crystallography constitute the major objective of this project.

Since these studies require large amounts of highly purified enzyme, the development of effective purification procedures is essential. Therefore, a major effort to improve more conventional purification procedures by developing new affinity chromatography methods has been undertaken.

The isolation of phenylalanine-tRNA synthetase from Salmonella typhimurium in particular is of interest for comparative studies with other phenylalanine-tRNA synthetases which have been isolated and characterized in a uniquely large number of different species and the great information about phenylalanine tRNA molecules.

The parallel studies on the characterization of the histidine-tRNA synthetase discussed in the last report have been continued.

Methods Employed: A large preparation of Salmonella bacteria (8 kg) was grown in the NIH fermenter unit. The cells were lyzed and phenylalanine tRNA synthetase activity was found in the 30-65% ammonium sulfate fraction by means of an improved enzyme assay. The subsequent partial purification employed DEAE-Sephadex-, hydroxylapatite- and Sephadex G-100 and G-200 chromatography. The enzyme was finally purified to homogeneity by means of affinity chromatography. For this purpose a resin was developed which contained the substrate phenylalanine linked to a butylamine-"arm" via its carboxyl group. Various chemical routes were used in an attempt to optimize the yield of phenylalanine coupling. Common to all approaches was the protection of the α-amino group with a protecting group which subsequently could be cleaved on the resin under conditions which do not affect the integrity of the Sepharose beads.

The characterization of the enzyme so far employed disc gel electrophoresis, SDS gel electrophoresis, analytical ultracentrifugation, amino acid analysis and absorption and fluorescence spectroscopy. Kinetic studies were performed by varying the concentration of one or the other component in the standard assay.

From the work described in the last report, the histidine-tRNA synthetase has been purified to homogeneity, its basic characteristic ascertained, and is now available for detailed studies of its interaction with its cognate t-RNA.

The preparation of large amounts of the phenylalanine and histidine tRNA species have been initiated by chromatographic fractionation of crude tRNA mixtures, from closely related E. coli, on reverse phase RPC-5 and BD-cellulose columns.

Major Findings: In the final step of the purification by means of affinity chromatography the phenylalanine enzyme was either retarded or completely retained by the resin depending on the degree of phenylalanine substitution on the agarose. In the latter case elution was performed with 0.5 molar potassium chloride. Non-specific absorption of contaminating proteins to non-reacted aminobutyl-"arms" was sometimes observed but could be eliminated by a preparation of the resin which was characterized by a high yield of the phenylalanine coupling reaction.

The homogeneity of the enzyme was demonstrated by analytical ultracentrifugation and disc-gel electrophoresis. A molecular weight of 210,000 has been established by means of equilibrium centrifugation and gel chromatography. In preliminary SDS electrophoresis experiments two bands corresponding to molecular weights of 53,000 and 100,000 have been found. It seems likely that the enzyme consists of four identical subunits, but additional experiments will be required for the clarification of the subunit structure. Several amino acid analyses of the enzyme have been performed.

The K_M values for the substrates phenylalanine and $tRNA^{Phe}$ were determined as 2.5×10^{-6} M and 1.0×10^{-6} M, respectively.

In preliminary binding studies the quenching of intrinsic protein fluorescence was employed for monitoring the binding process of the substrates. In case of ATP a total quenching of 5.2% was observed and its analysis allowed the estimate of an affinity constant of 8×10^4 M^{-1}. With the substrate phenylalanine a total quenching of less than 2% resulted which could not be analyzed in terms of an affinity constant. The fluorescence binding studies with these two substrates will have to be complemented by equilibrium dialysis experiments.

The basic characterization of the subunit structure, amino acid composition and enzymatic properties of the histidine-tRNA synthetase has been accomplished and now published.

For both enzymes the availability of large amounts of their cognate tRNA are necessary for the next steps in the analysis of the interactions of the protein with the nucleic acid and other ligands. The preparation of the ...e tRNA has been begun by the chromatographic procedures noted above and has been successful but the amounts available are still too small for the chemical and physical studies envisaged.

Significance to Biomedical Research: The affinity chromatography method developed for the purification of phenylalanine-tRNA synthetase from Salmonella typhimurium may well be suitable for the purification of other aminoacyl-tRNA synthetases from various species. An even broader application of the method can be envisaged for the purification of other enzymes which recognize and interact with a specific amino acid as their substrate, inhibitor or effector.

The partial characterization of this enzyme represents an initial step in the study of the structure and mechanism of these proteins which are important in the mechanism of biosynthesis of proteins in the cell and, possibly, in regulatory mechanisms.

The on-going related studies of the histidine-tRNA synthetase are of comparable significance and goal.

Proposed Course of Project: The major focus will be on the tRNA-enzyme interactions whereby mainly fluorescence techniques and NMR-spectroscopy will be employed. It is hoped that aside from the overall-association constant a more detailed picture of this highly specific interaction will eventually emerge from these studies. NMR studies should reveal possible hydrogen bonds formed between the tRNA molecule and the enzyme and also allow the detection of a possible conformational change of this ligand due to the binding. It is hoped that from such studies general inferences can be made about the interaction of proteins with nucleic acids.

Publications:

DeLorenzo, F., DiNatale, P. and Schechter, A.N.: Chemical and physical studies on the structure of the histidyl-tRNA synthetase from Salmonella typhimurium, J. Biol. Chem. 249, 908-813, 1974.

Schiller, P. and Schechter, A.N.: Phenylalanine-tRNA synthetase from Salmonella typhimurium. In Methods in Enzymology: Enzyme Purification, Part B, Affinity Techniques (Jakoby, W.B. and Wilchek, M., eds.), in press.

Serial No. NIAMDD-LCB-17
1. Lab. of Chemical Biology
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Indivudual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of complex formation between bovine neurophysin and the neurohypophysial peptide hormones, oxytocin and [8-lysine] vasopressin.

Previous Serial Number: NIAMDD-LCB-9

Principal Investigator: John H. Griffin

Cooperating Units: NICHD, RRB (Jack S. Cohen), French Atomic Energy Commission (Paul Cohen, Robert Alazard)

Man Years:

Total: 0.2
Professional: 0.2

Project Description:

This project was terminated by the departure of Dr. Griffin from the NIH.

Publications:

Griffin, J.H., Cohen, J.S., Cohen, P., and Carrier, M.: Proton magnetic resonance studies of hormonal interactions at the molecular level: complex formation between bovine neurophysins and oxytocin. J. Pharm. Sci., in press.

Griffin, J.H., Alazard, R., and Cohen, P.: Complex formation between bovine neurophysin I and oxytocin, vasopressin, and tripeptide analogs of their NH_2-terminal region. Absorbance and circular dichroism difference spectroscopic studies. J. Biol. Chem. 248, 7975-7978, 1973.

Serial No. NIAMDD-LCB-18
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Regulation of histidine biosynthesis in Salmonella typhimurium.

Previous Serial No.: NIAMDD-LCB-20, 21, 22

Principal Investigator: Robert F. Goldberger

Other Investigators: Marilyn Meyers
Roger Deeley
Kathleen Mullinix
Henry Kronenberg
John Kovach
Mark Levinthal
Carmello Bruni

Man Years:

Total: 2.0
Professional: 1.8
Other: 0.2

Project Description:

Objectives; To elucidate the mechanism by which expression of the histidine operon is regulated in response to the needs of the microorganism under a variety of conditions. Previous work suggested that this system is autogenously regulated--that is, experesssion of the genes of the operon is regulated by a protein specified by one of those very genes. The protein in question is known to also function as an enzyme, the so-called G enzyme that catalyzes the first step in the pathway for histidine biosynthesis. A role for this enzyme as a regulatory protein that controls experession of the histidine operon is indicated by the findings of several different projects.

Methods Employed: Genetic and biochemical methods employed are clarified in the following section.

Results:
1) Strains have been isolated in which a single mutation in the structural gene for the G enzyme leads not only to synthesis of structurally damaged G enzyme but also to loss of regulation of the entire histidine operon.
2) The G enzyme has been shown to interact with high affinity and high specificity with aminoacylated histidyl-tRNA. Such properties fulfill an important requirement for any regulatory protein that may influence expression of the histidine operon. The enzyme does not, however, differentiate between

normal (wild type) histidyl-tRNA and immature (hisT) histidyl-tRNA. This finding eliminates the possibility that the difference in the regulatory properties in the two species of tRNA is mediated by G enzyme recognition and suggests that the difference is mediated by the interaction of a G enzyme-tRNA complex with the DNA of the histidine operon.

3) The G enzyme has been found to interact specifically with the DNA of the histidine operon carried in the genome of a defective ϕ80 transducing bacteriophage. Furthermore, competition experiments, using various preparations of radioactively labeled and unlabeled DNA suggest that the enzyme interacts with the regulatory region of the operon.

4) A purified in vitro system was developed for transcribing the histidine operon carried in the genome of a defective ϕ80 transducing bacteriophage. Messenger-RNA specific for the histidine operon was found to be synthesized in this system from the correct ("sense") strand of the phage DNA. This mRNA had a sedimentation constant identical with that of histidine operon mRNA synthesized in vivo. Addition of purified G enzyme to the in vitro system caused a severe and specific inhibition of transcription of the histidine operon, demonstrating that the enzyme functions as a negative control element for the histidine operon in vitro and suggesting that it plays a similar role in vivo.

5) Studies are under way to develop an in vitro system for synthesizing the enzymes of the histidine operon from the DNA of a defective ϕ80 transducing phage that carries the operon in its genome. Because the in vitro system involves both E. coli and S. typhimurium, it is complicated by the problem of the bacterial restriction-modification systems. For this reason, a good deal of effort has gone into obtaining "restrictionless" strains and into other possible ways to solve the restriction problem.

Significance to Biomedical Research: An understanding of the molecular mechanisms involved in regulation of gene expression lies at the root of many problems in the biomedical sciences.

Proposed Course of Research: The projects described herein were transferred together with the personnel to the NCI.

Publications:

Blasi, F., Bruni, C.B., Avitabile, A. and Deeley, R.G., Goldberger, R.F., and Meyers, M.: Inhibition of transcription of the histidine operon in vitro by the first enzyme of the histidine pathway. Proc. Natl. Acad. Sci. U.S.A. 70, 2692, 1973.

Avitabile, A., Bruni, C.B., Carlomagno, M.S., Meyers, M., Vigliar, G. and Blasi, F., J. Bacteriol. 114, 351, 1973.

Goldberger, R.F., Deeley, R. and Mullinix, K.P.: Regulation of gene action in procaryotic cells. In Caspari, E. and Ravin, A.W. (eds.) Genetic Organization, Vol. II, Academic Press, New York, 1974.

Serial No. NIAMDD-LCB-19
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Purification of the first enzyme of the histidine operon by affinity chromatography.

Previous Serial Number: NIAMDD-LCB-18

Principal Investigators: Loren G. Lipson
Robert F. Goldberger

Man Years:

Total: 0

Project Description:

This project has been terminated.

Serial No. NIAMDD-LCB-20
1. Lab. of Chemical Biology
2. Biosynthesis and Control
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Correlation of cross reacting material with enzyme activity and mutant type of the first enzyme of the histidine operon (the G enzyme).

Previous Serial Number: NIAMDD-LCB-19

Principal Investigators: Loren G. Lipson
Robert F. Goldberger

Man Years:

Total: 0

Project Description:

This project has been terminated.

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL PHARMACOLOGY

I. PROTEIN STRUCTURE AND MECHANISM OF ENZYME AND HORMONE ACTION

Aminoacyl tRNA Synthetases

Over a period of years we have developed a hypothesis that the aminoacyl tRNA synthetases of yeast can exist in two forms. One is very labile and requires an activator. The other is more stable and works well without any activating substance, but it can be converted to the labile activator-requiring form by inorganic sulfide. New experiments with yeast suggest that both forms are present in the cells, and the activating substance can vary in amount independently of the enzymes.

(Dr. S. Black and Mrs. B. Hazel)

Histidine Ammonia-Lyase

I. Mechanism of Action. Studies of the mechanism of the α,β-elimination of NH_3 from histidine and 4-fluorohistidine by histidine ammonia-lyase have been extended to 4-NO_2-histidine, 2-NH_2-histidine, and 2-fluorohistidine. Steady state and transient state kinetic studies have confirmed our observation that the rate limiting step precedes the dissociation of the amino enzyme intermediate, the last step common to all the substrates analyzed. The C-H bond cleavage determines at least partially the reaction rate with histidine and 4-fluorohistidine, but not 4-NO_2-histidine.

The urocanic acid analogs obtained by deamination of the compounds mentioned above were tested as substrates or inhibitors of the next enzyme in the degradative pathway of histidine, urocanase. 2-Fluorourocanate was found to be a very potent competitive inhibitor with a K_i of 10^{-7} M (K_m for urocanate = 0.5×10^{-4} M) and is therefore a very useful tool to study the mechanism of action of this enzyme which contains α-ketobutyrate as a prosthetic group.

(Drs. C. B. Klee, L. A. Cohen [A:LC], K. L. Kirk [A:LC], and P. McPhie [A:LMB])

II. Active Site Studies. The nature of the prosthetic group at the active center of histidine ammonia-lyase has been investigated further using the stereospecific inactivation of the enzyme by [^{14}C]- or [^{35}S]L-cysteine. This modification appears to be active site specific since it is prevented by the substrate L-histidine, but not by D-histidine. A labeled peptide with the 340 nm absorption peak characteristic of the inactivated enzyme has been purified from a pronase digest of the modified enzyme. Further fractionation and characterization of this peptide is being carried out in order to elucidate the chemical nature of the 340 nm absorbing residue which is believed to be a derivative of the prosthetic group.

(Dr. C. B. Klee)

Studies Concerning the Mechanism of Exophthalmos and of Thyrotropin Receptor Function

We have demonstrated that thyrotropic hormone is exophthalmogenic but that partial pepsin digestion could yield an exophthalmogenic fragment of the thyrotropin molecule which has lost its thyroid stimulating ability. Studies concerning the binding of these hormonal factors and their biochemical effects on retro-orbital and thyroid tissues led us to suggest that exophthalmos is a two-factor disease requiring the simultaneous presence of thyrotropin (TSH) or a TSH derivative without thyroid stimulating ability, and of an abnormal gamma globulin which is not a long acting thyroid stimulator (LATS) and which can be detected in the serum of patients with exophthalmos. The TSH or the TSH derivative is presumed to be the direct effector; the abnormal gamma globulin is presumed to target this effector by modifying its binding characteristics to retro-orbital receptors. The finding that cellular sensitivity (as measured by leucocyte migration assays) preceded the appearance of the abnormal serum gamma globulin has resulted in the use of this assay to detect Graves' disease, patients who will get exophthalmos during treatment of their hyperthyroidism. This detection method and the use of nontoxic doses of Imuran have led to the prevention of this disease in a preliminary series of 400 patients.

Experiments during this year have been aimed at understanding the different nature of the TSH-receptor interaction in retro-orbital and thyroid cell membranes. The accomplishments of these experiments can be summarized as follows: (1) The development of procedures to iodinate TSH or its exophthalmic fragment without losses in biological activity. (2) The demonstration of similar comparative binding properties in thyroid and retro-orbital tissues using iodinated hormone preparations. (3) The solubilization and partial purification of TSH receptors from thyroid and retro-orbital membranes. (4) The creation and isolation of low molecular weight receptor material. (5) The demonstration by comparative binding and biological assay procedures that neither the α or β subunits of TSH can account for TSH activity, that this is a consequence of poor binding, that the β TSH subunit carries the determinants for bioactivity, and that the α subunit imposes conformational changes on the β subunit which promote binding and bioactivity commensurately in TSH but only binding in LH.

(Drs. L. D. Kohn, R. L. Tate, R. J. Nossal [CR:PSL], J. Wolff [A:CE], and R. J. Winand [University of Liege, Liege, Belgium])

Studies Concerning the Biosynthesis of Collagen

Dermatosparaxis is an inherited connective tissue disorder of cattle which is characterized by extreme fragility of the skin and an accumulation in the dermis of procollagen, the triple helical precursor of collagen which is composed of pro α1 and pro α2 chains. We have shown that pro α1 and pro α2 chains are ribosomal products which contain an additional peptide of 100 to 200 residues at the amino termini of their respective α chains. In addition, we have described an enzyme activity in normal bovine tissues which excised the amino terminal extension of the precursor chains to yield electrophoretically normal chains. This activity is extracellular and is absent or markedly reduced in

dermatosparaxis tissues and in fibroblast cultures of several patients with variants of the Ehlers-Danlos syndrome, *i.e.*, the accumulation of procollagen and the resultant tissue changes were apparently caused by a defect in a specific enzyme, "procollagen peptidase."

We have described the purification of calf tendon procollagen peptidase, have characterized several of its properties, and have shown that the enzyme is an endopeptidase which excises the amino terminal peptide of procollagen in block rather than in multiple fragments. We have developed immunological assays of procollagen and procollagen peptidase which have allowed us to study the synthesis of these molecules in fibroblast cultures. The conclusions of the present studies can be summarized as follows: (1) Procollagen is a ribosomal product which is packed in a triple stranded array in vesicles within the cell and excreted as such. (2) Procollagen peptidase activity is maximal in the extracellular fluid only after a time lag during which time an inactive enzyme precursor is demonstrable. (3) Cells in culture secrete two forms of procollagen, one of them being a fetal procollagen which can be isolated from amniotic fluid. (4) Amniotic fluid and fetal sera contain large amounts of the excised propeptide and of fetal procollagen.

(Mr. George Lee [American University], Drs. L. D. Kohn, L. W. Tice [A:LEP], C. M. Lapiere [University of Liege, Liege, Belgium], G. R. Martin [D:LB], and E. C. Leroy [Columbia University College of Physicians and Surgeons, New York, New York])

Studies Concerning Membrane Structure and Function

The membrane-bound D-lactate dehydrogenase of *E. coli* ML 308-225 has been solubilized and purified to homogeneity. Recent experiments demonstrate that guanidine extracts from wild type vesicles containing D-lactate dehydrogenase activity are able to reconstitute D-lactate-dependent oxygen consumption and active transport in membrane vesicles from an *E. coli* mutant deficient in D-lactate dehydrogenase. Present experiments have shown that purified enzyme can reconstitute transport. The reconstituted vesicles are different from native in having their enzyme accessible to extracellular inhibitors such as an anti-enzyme sera.

The membrane-penetrating MN-glycoprotein of the human erythrocyte has been further examined. Circular dichroism data analyzed by curve fitting techniques show that the intramembranous region of this glycoprotein forms an unusually stable helix when incorporated into egg lecithin liposomes. A computer search for hydrophobic regions in protein sequences suggests that the intramembranous domain of the MN-glycoprotein is exceptionally hydrophobic. These data support our previous contention that in the absence of strong perturbations, this intramembranous peptide segment should be helical in the membrane *in situ*. The length of this helix (35 Å) is of the order of thickness of the hydrophobic core of the membrane.

When isolated by trypsin digestion, the intramembranous segment contains bound cholesterol and phospholipid which can be removed with organic solvents.

In addition, this segment contains tightly bound phosphorous which is not associated with phospholipid.

(Drs. L. D. Kohn, H. R. Kaback [Roche Institute of Molecular Biology, Nutley, New Jersey, and J. P. Segrest)

Studies Concerning Structure and Function of Oxido Reductases

Oxido reductases which convert glyoxylate, hydroxypyruvate, tartrate, and malate to glycolate, D(-)-glycerate, oxaloglycolate, and oxaloacetate, respectively, have previously been purified to homogeneity and characterized. Experiments have been directed towards understanding their mechanism of action, the mechanism by which they achieve specificity, and the mechanism by which their subunits associate to yield active enzyme. Present experiments involve sequence procedures of the active site of the induced and constitutive hydroxypyruvate reductases.

(Dr. L. D. Kohn)

Studies Concerning Centrifugation

As a cooperating unit, ultracentrifugation studies have been performed this year for the following laboratories of the NIAMDD: the Laboratory of Biochemistry and Metabolism (Drs. G. G. Ashwell, E. Cabib, and W. B. Jakoby), the Laboratory of Chemistry (Drs. C. R. Creveling and P. Torrence), the Laboratory of Biophysical Chemistry (Dr. D. R. Kominz), the Laboratory of Chemical Biology (Dr. A. N. Schechter), and the Clinical Endocrinology Branch (Dr. K. Ingham). Collaborative experiments with Drs. E. W. Miles and O. Adachi of this laboratory have shown that native $\alpha_2\beta_2$ and synthetic $\alpha_2\beta_2$ tryptophan synthetase have the same physical properties.

(Dr. L. D. Kohn)

Tryptophan Synthetase of *Escherichia coli*

Tryptophan synthetase is a multienzyme complex with an $\alpha_2\beta_2$ subunit structure. The β_2 subunit contains a cofactor, pyridoxal-P, which serves as a useful optical probe in studies of the interactions of the α and β_2 subunits and in studies of the structure and function of the β_2 subunit and the $\alpha_2\beta_2$ complex.

New rapid procedures for the purification and crystallization of the β_2 subunit and the $\alpha_2\beta_2$ complex have been developed which give high yields of high activity enzymes. The molecular weight and subunit composition of both native and synthetic crystalline $\alpha_2\beta_2$ complexes have been determined by ultracentrifuge studies. Large crystals of the complex are being grown which may be suitable for x-ray crystallographic studies. The interaction of the α and β subunits is being studied by circular dichroism and absorption difference spectroscopy.

Studies on the mechanism and active site of the β_2 subunit have continued. Pyridoxal-P has been found to sensitize the photooxidation of one specific,

essential histidyl residue per β monomer. This essential histidyl residue has been located in the region of the β_2 subunit which binds pyridoxal-P. These findings show that the photosensitive histidyl residue is in the catalytic site of the β_2 subunit.

(Drs. E. W. Miles and O. Adachi)

II. SYNTHESIS OF PROTEINS AND NUCLEIC ACID

Nucleic Acid Synthesis

Replication of linear duplex T4 DNA *in vivo* requires several proteins encoded by the phage DNA including the T4 DNA polymerase and a DNA unwinding protein (gene 32 protein). The purified T4 DNA polymerase by itself cannot synthesize DNA using a nicked linear duplex DNA template *in vitro*, apparently because it is unable to displace the DNA strand which is annealed to the strand being copied. We have previously shown that the polymerase will copy such a template in the presence of the gene 32 DNA unwinding protein, which promotes strand displacement. We have now shown that the major product of this reaction is a rapidly renaturable, branched DNA molecule with a base composition like that of the template. At least part of this product is covalently attached to the DNA serving as the template-primer. The results suggest that the polymerase begins synthesis at a 3' hydroxyl terminated nick in the duplex DNA, using the nicked strand as the primer and the complementary strand as the template. The 32 protein facilitates this copying by displacing the strand previously annealed to the strand serving as the template. The rapidly renaturable hairpin structures are then formed when the polymerase stops copying the template strand and copies instead either the displaced strand or the newly synthesized DNA which is attached to the primer. Presumably this switching away from the original template is prevented *in vivo* by one or more of the other proteins required for T4 DNA replication.

The alternating polymer, poly[d(A-T)], is also formed to a variable extent during the later stages of the nicked duplex DNA primed reaction with the polymerase and the 32 protein. It probably results from the creation of a poly[d(A-T)] template by continuous strand slippage after the copying of a region of the DNA with a short stretch of alternating A and T residues.

The protein product of T4 phage gene 44 is required for T4 DNA replication *in vivo*. This protein has been extensively purified using the stimulation of DNA synthesis in an extract of cells infected with a gene 44 mutant as an assay. The purified protein has no detectable polymerase, nuclease, or deoxynucleotide triphosphatase activities. We are presently trying to find conditions under which the gene 44 protein will stimulate DNA synthesis catalyzed by the T4 DNA polymerase and the gene 32 DNA unwinding protein.

(Dr. N. G. Nossal)

Factors contributing to the fidelity of DNA replication can be studied with the T4 DNA polymerase, since some temperature sensitive mutations in the

structural gene for this enzyme, at permissive temperatures show either an increased or a decreased rate of mutation of markers in other genes. We have compared the highly purified DNA polymerases produced after infection with wild type, mutator (*ts* L88), or antimutator (*ts* CB120) T4 phage, using the homopolymers poly dA · poly dT as a model system for studying the frequency of *in vitro* copying errors. The noncomplementary nucleotides dGTP and dCTP are utilized more frequently by the mutator and less frequently by the antimutator compared with the wild type polymerase. The utilization of noncomplementary nucleotides was measured by the DNA dependent production of deoxynucleoside monophosphate from triphosphate. There was no measurable stable incorporation of the wrong nucleotide, apparently because the incorporated noncomplementary nucleotides are so efficiently removed by the 3' to 5' exonuclease activities associated with each polymerase. Thus with this template the three enzymes appear to differ in their frequencies of incorporation of noncomplementary nucleotides. No differences in the rates of removal of erroneous nucleotides could be demonstrated under these conditions, although the antimutator polymerase consistently hydrolyzed a larger fraction of newly incorporated complementary nucleotides than the wild type or mutator enzymes. We have not yet found any qualitative difference in the type of errors made by the three proteins.

The alternating polymer, poly[d(A-T)] which has secondary structure due to intrastrand hydrogen bonding has been used to study the interaction of the mutant polymerases with the gene 32 DNA unwinding protein. Both the mutator and antimutator enzymes copy poly[d(A-T)] very poorly compared with the wild type enzyme. The DNA unwinding protein stimulates synthesis with the mutator enzyme only 2-fold. In contrast, it stimulates synthesis with the antimutator polymerase to an even greater extent than it stimulates the wild type enzyme. Thus the *ts* CB120 antimutator polymerase appears to be defective in its ability to copy a template with intrastrand secondary structure.

(Dr. F. D. Gillin)

Elongation Factors

Work from other laboratories has shown that three elongation factors are obtainable from extracts of *E. coli* and are involved in ribosomal dependent peptide chain elongation *in vitro*: Tu, Ts, and G. (Tu and Ts are usually obtained as a complex, Tu-Ts, called T.) Both T (*i.e.*, Tu-Ts) and G are required for polypeptide synthesis. However, earlier work from this laboratory and others demonstrated the existence of yet another elongation factor (or another form of the elongation factors) which had the property of promoting peptide chain elongation by itself, *i.e.*, it did not require the addition of T or G and was called (G+T). Work from other laboratories had shown that this factor was not separable into T and G by methods that were shown to resolve these two activities, and (G+T) could not be produced by mixing previously separated T and G. Furthermore, work from this laboratory suggested that although (G+T) was stabilized by the proteolytic inhibitor, phenylmethylsulfonyl fluoride, and could be partially purified in its presence, we did note that the inhibitor did not completely prevent the loss of (G+T) especially during chromatography on DEAE-Sephadex. It thus seemed possible that

the failure of others to observe (G+T) could be due to the instability of this factor on DEAE-Sephadex, and accordingly we set out to purify the elongation factors without using this chromatographic medium. In the course of this work, as well as in our earlier work, it was noted that material with T activity and G activity behaved quite heterogeneously during fractionation. Therefore, methods were developed to identify which of the protein bands seen on disc gel electropherograms correspond to Tu, Ts, and G, and thereby it was possible to monitor all of the fractions obtained during the purification of these factors for their content of the various elongation factors. In this way a comprehensive description of the state and nature and relative amounts of the elongation factors present in ribosome-free extracts of *E. coli* was made.

Before presenting these results it is important to point out that polypeptide synthesis was used as the functional assay for the elongation factors, and it was found quite early that Tu alone as well as Tu-Ts (T) could complement G in carrying out peptide chain elongation *in vitro*; *i.e.*, Ts is not absolutely required, at least *in vitro*. (This dispensability of Ts has also been shown by others.) At any rate for polypeptide synthesis at least, fractions exhibiting at least "T activity" can contain either Tu alone or Tu-Ts (*i.e.*, T). The salient features of the results then are as follows.

(1) The material with the activity and chromatographic properties of (G+T) is either a very weak complex of G and Tu or an admixture of Tu and G resulting from their fortuitous cochromatography on various media.

(2) Tu exists in the cell as either free Tu, as bound to Ts (*i.e.*, as T), or as loosely aggregated with G or some other cellular component(s). To be certain that three three Tu's were in fact the same polypeptide, cells were labeled with [^{14}C]arginine and peptide maps were prepared from the tryptic digests of free Tu, the Tu bound to Ts, and aggregated Tu. The peptide maps of all three were identical and corresponded well with the number of arginines determined by amino acid analysis.

(3) In the course of this work, methods were developed for determining directly the amounts of radioactive Tu and G in cell extracts, and it was found that for every mole of ribosomes there is about 1 mole of G (in agreement with what others have found) and about 9 moles of Tu. This is substantially more (in fact, 9 times more) Tu than was previously thought to be present in cells, and the bulk of the Tu appears to be free Tu, *i.e.*, not bound to Ts in the form of T or aggregated to other molecules.

(Dr. A. V. Furano)

Methylamino Acids

A comparative study of the methylated amino acids in culture 3T3 mouse cells and *E. coli* was made. Cells were labeled with [^{14}C-methyl]methionine and then fractionated into crude pellet (which consists mainly of nuclei with the mammalian cells and membranes with the bacterial cells), ribosome-free cytoplasm, small ribosomal subunit (40S for the mouse cells and 30S for the bacteria), and large ribosomal subunit (60S for the mouse cells and 50S for

the bacteria). Portions of these fractions were hydrolyzed and their content of methylamino acids were analyzed by 2-dimensional thin layer chromatography. As had been shown for methylated nucleic acid bases, the bacterial extracts contained smaller amounts and fewer kinds of methylated amino acids than did the mammalian extracts. The ribosome-free cytoplasm and pellet obtained from *E. coli* revealed only small amounts of N,N'-dimethylarginine.

The 30S subunit also contained N,N'-dimethylarginine and three as yet unidentified spots, one of which might be a methylated histidine. The 50S subunit showed a more complex pattern which except for N,N'-dimethylarginine and one of the unidentified spots was distinct from that shown by the 30S subunit. The 50S subunit contained dimethyllysine, methylysine, a spot tentatively identified as trimethyllysine, and an unidentified spot, in addition to the unidentified one it shares with the 30S subunit.

The patterns obtained from the 3T3 cells were more complex and appeared to contain larger amounts of the methylated amino acids. The nuclei-containing pellet contained only those methylated amino acids that have been described for histones and in fact showed essentially the same pattern as that obtained from the hydrolysate of [^{14}C]methyl-labeled histone prepared from 3T3 cells; Mono-, di-, and probably trimethyllysine, N,N-dimethylarginine (or N-methylarginine) were the predominant amino acids identified. The ribosome-free cytoplasm had an essentially similar pattern except that it lacked methyllysine and contained the N,N'-dimethylarginine that was not present in the pellet. The 40S subunit contained N,N-dimethylarginine (or N-methylarginine) as its most prominent methylated amino acid and also a spot which as in the smaller subunit of *E. coli* was tentatively identified as a methylhistidine. The presence of these two spots distinguished the 40S from the 60S which contained small amounts of mono-, di-, and probably trimethyllysine, N,N'-dimethylarginine, and an unidentified spot which was common to the 30S and 50S subunits of bacteria.

In summary, the cytoplasm and crude pellet of *E. coli* (which has been shown to lack histones) contain only small amounts of methylamino acids. Except for an unidentified spot on the 30S and a methylarginine (N,N-dimethyl or N-methyl) on the 40S, the small ribosomal subunits of bacteria and mouse are similar in pattern, if not amount. Except for one unidentified spot in the bacterial 50S subunit, the large ribosomal subunits are similar in pattern. The mouse ribosome-free cytoplasm contains large amounts and more types of methylated amino acids than the ribosome-free cytoplasm of bacteria.

(Drs. A. V. Furano and M. Klagsbrun [Children's Hospital Medical Center, Boston, Massachusetts])

III. COMPLEX CARBOHYDRATES

Affinity Columns for the Purification of Antibodies Directed against Carbohydrates

A simple method has been devised for the attachment of oligosaccharides

to Sepharose for the preparation of affinity columns. ω-(p-Aminophenyl)-alkylamines are used as follows. The sugar is first reacted with the alkylamine moiety of the molecule to form n-alkylglycosylamines which are reduced to stable secondary amines with borohydride. The arylamine moiety is then reacted with cyanogen bromide-activated Sepharose to effect the coupling. The yield of sugar coupled in terms of starting oligosaccharide is over [illegible]%.

(Drs. V. Ginsburg and A. M. Jeffrey)

Synthetic Carbohydrate Antigens

Synthetic antigens have been produced by coupling oligosaccharides isolated from human milk to polylysine. The procedure for coupling involves the oxidation of the sugars to aldonic acids and then acylation of the amino groups by the aldonic acids by a modification of a mixed anhydride method. The conjugates produced are immunologically specific.

(Drs. V. Ginsburg and D. A. Zopf)

IV. CELL SURFACES

Structure and Assembly of *Escherichia coli* Membranes

The surface of *E. coli* consists of an inner, or cytoplasmic, membrane, a rigid cell wall, and an outer membrane. All the components of the outer membrane are synthesized elsewhere in the cell: protein on ribosomes within the cell; lipid and lipopolysaccharide (LPS) in the inner membrane. Our goal is to define how these components are assembled during outer membrane biogenesis and organized in the completed membranes.

Previous work in this laboratory using density labeling of LPS indicated that there were about 10 to 30 specific insertion points in the outer membrane, into which newly-synthesized LPS was translocated from the inner membrane, and from whence it spread to the remainder of the outer membrane. Work in another laboratory (Dr. P. Mühlradt's) has now confirmed this finding by another technique, electron microscopic visualization of ferritin-labeled antibody to newly-synthesized LPS. His experiments seem to suggest that the newly-synthesized LPS might be emerging into the outer membrane through bridges between the two membranes. The outer membrane above such bridges is known as the site of adsorption of several phages, including phage BF23. Since recently the receptor for this phage has been found to be the same as the receptor for cyanocobalamin, we decided to see whether the insertion points for LPS, isolated by our technique, contained higher concentrations of cyanocobalamin receptor, as measured by extent of binding of radioactive cyanocobalamin. Experiments to date indicate no higher binding of cycanobalamin by the insertion points as compared to the remainder of the outer membrane, although the finding of another laboratory that receptor is present only in the outer membrane was confirmed.

(Drs. P. Bhattacharyya and L. Leive)

When the density gradient technique of separating insertion points from the remainder of the outer membrane was used to prepare large quantities of each for analysis, it became apparent that the technique did not provide sufficient resolution for complete separation of the two types of membrane fragments. For this reason another method of separation was sought. LPS is a good antigen and antisera prepared by LPS injection are specific for the carbohydrate terminus of the molecule. When galactose is added to a UDP-galactose epimerase-less mutant, it instantly changes from production of one antigenic type (1) of LPS to another (2). By adsorbing sera from rabbits injected with type 2 LPS with either whole cells or isolated LPS of type 1, it is possible to prepare antibody highly specific for only type 2 LPS. Such antibodies were prepared and coupled to Sepharose, and used for affinity chromatography. Labeled type 2 LPS was shown to adsorb to the column and to be specifically eluted with nonradioactive type 1 LPS or with carbohydrate haptene produced from such LPS. In preliminary experiments, outer membranes prepared from the mutant have been shown to adsorb to the column if, and only if, the mutant is grown in the presence of galactose (producing type 2 antibody) and not if it is not grown in the absence of galactose (producing type 1 antibody). When adsorbed, such membrane fragments can be eluted by type 2 LPS or haptene. When perfected the technique will be used for fractionation of outer membrane fragments from cells that have just begun production of type 2 LPS, in an effort to isolate outer membrane insertion point fragments, which are enriched for new LPS.

(Dr. L. Leive)

V. MECHANISMS OF INHERITANCE IN SACCHAROMYCES CEREVISIAE

The "Killer Character" of *Saccharomyces cerevisiae*

The "killer character" of the eukaryote *Saccharomyces cerevisiae* is an extrachromosomal genetic element which is associated with two discrete double-stranded RNA species and virus-like particles.

Mutants have been isolated in two chromosomal genes whose functions are essential for maintenance of the extrachromosomal killer genome (mak). One of these (mak-1) is located within 2 centimorgans of the centromere of chromosome XV. Two other chromosomal genes are needed for expression of the killing function (kex). One of these (kex-2) is located on chromosome XIV by disome analysis, but is not meiotically linked to any of the known markers on chromosome XIV. Another gene (rex) is needed to express resistance to the extracellular killer substance, and since mutants in this gene still express killing, the cells can commit suicide. Both kex and rex mutants still harbor the complete cytoplasmic killer genome.

Mutations in the extrachromosomal killer genome have also been obtained. One type has lost killing ability but retains resistance to killing and is recessive to wild type. A second type of mutant has lost both killing ability and resistance and is recessive to wild type, but introduction of the wild type killer plasmid results in selective loss during mitosis of the wild type

genome and retention of the mutant plasmid. This phenomenon is called "suppressiveness." A third type of mutant may lack killing ability or resistance or both and is recessive to the wild type. It has the interesting property that mating with any strain, even one apparently lacking the plasmid, restores the wild type phenotype. This suggests that these mutants carry the plasmid in some sort of "latent" state.

It has also been found that growth of killer strains at elevated temperatures results in the loss of part or all of the plasmid genome.

(Dr. R. B. Wickner)

DNA Replication in *Saccharomyces cerevisiae*

Mutants of yeast have been selected capable of dTMP uptake and incorporation specifically into DNA. Since DNA cannot be specifically labeled in yeast by either thymine or thymidine, these strains will be of great value in the study of DNA metabolism of yeast. These strains also incorporate dBrUMP into DNA. These mutants fall into four complementation groups. One group, tup-1, located on chromosome III, shows abnormal mating behavior when in conjunction with the α mating type. In addition homozygous a/α tup-1/tup-1 strains fail to sporulate.

(Dr. R. B. Wickner)

VI. POLYAMINES

The work in this area has largely centered around the compound, glutathionylspermidine, which we have isolated from *E. coli* at the beginning of stationary phase. The importance of this compound is indicated by quantitative assays showing that all of the bacterial spermidine and half of the intracellular glutathione are found in this form under these growth conditions.

Methods have been developed for the convenient isolation of relatively large amounts of glutathionylspermidine from *E. coli* and for its determination in the amino acid analyzer. Additional structural studies have shown that the glutathione is linked to the N^1-position of the spermidine.

Other related studies have been concerned with the substrate specificity and products of the *Serratia* spermidine dehydrogenase and on gas chromatographic methods for the polyamines. Several modifications have also been introduced into our automated method for the determination of amines in order to separate the amines from their methylated derivatives.

(Drs. H. Tabor and C. W. Tabor)

Studies have been started on the content of the polyamine synthesizing enzymes in various mutants of *E. coli*.

(Drs. C. W. Tabor, E. W. Hafner, and H. Tabor)

VII. EVOLUTION

Experiments on the origin of the genetic code have been continued. One development is a finding that 5-isobutyluracil has a significantly greater affinity for a peptide containing leucine than for analogous peptides with phenylalanine and valine. The implication is that the isobutyl side chain of leucine interacts with some specificity with the corresponding group of the uracil derivative, so that the latter may be a "primitive codon" for leucine.

(Drs. S. Black and H. M. Chao)

VIII. TRAUMATIC SHOCK AND TRANSPLANTATION

Studies on the role of histamine in burn, tourniquet, and endotoxin shock suggest that histamine protects against burn shock via its action on both H_1- and H_2-histamine receptors. In tourniquet and endotoxin shock, however, histamine produces deleterious effects as a consequence of its action on H_1-histamine receptors.

Measurement of the effect of age on transvascular fluid movement by the application of a mild vacuum to the ear or tail of mice demonstrated a large decrease of swelling which correlated with growth. This result was not due to changes in subcutaneous pressures or compliance. Other experiments were carried out on the influence of hormones on fluid movement.

Experiments to prolong the survival time of allogenic skin grafts by placing donor grafts in tissue culture media for 7-10 days prior to their application on recipient animals, as reported by Summerlin, were unsuccessful.

(Drs. K. Markley and S. M. Rosenthal and Mrs. E. T. Smallman)

IX. MYCOBACTERIUM

Continued time lapse cinemicrographic studies on the growth of granulocytes in our culture system revealed that a total of three successive crops of granulocytic colonies appeared in a period of 18 days. Multiplication and maturation of granulocytes were observed on top of the feeder cell of the colonies. The cytochemistry of the feeder cell was studied.

New techniques were developed for long-term cultivation of macrophages obtained from various tissue and sites of the mouse, including blood, alveolar washings, lungs, spleen, fetal liver, thymus, lymph nodes, cardiac muscle, and subcutaneous tissue. Motion pictures of these cells were taken.

More drugs were tested in murine leprosy using our new chemotherapeutic model, *i.e.*, using the survival time of CBA/J mice as the criterion for drug evaluation.

(Dr. Y. T. Chang)

Serial No. NIAMDD-LBP-1
1. Biochemical Pharmacology
2. Biochemistry of Amino Acids
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Biochemistry of Sulfur-Containing Compounds

Previous Serial Number: Same

Principal Investigator: Dr. Simon Black

Other Investigators: Dr. Helen M. Chao and Mrs. Blondel Hazel

Cooperating Units: None

Man Years:

Total:	4.2
Professional:	3.0
Others:	1.2

Project Description:

Objectives:

A long term objective is discovery of enzymatic and regulatory mechanisms involved in the synthesis of constituents of living tissue, particularly of proteins.

Methods Employed:

Chemical and enzymatic techniques involving the use of spectrophotometry and radioactive substances are evolved to meet the needs of special problems as they arise.

Major Findings:

I. Protein Synthesis. The aminoacyl tRNA synthetases of all living organisms catalyze the following general reaction:

amino acid + ATP + tRNA $\rightleftharpoons$ aminoacyltRNA + AMP + PP.

We have found previously that a number of these enzymes from several sources are sensitive to inorganic sulfide, their activity in the presence of this agent becoming completely dependent upon activator-like substances such as an alkali-denatured protein and glutathione. In the past year, our attention has largely centered on showing that within the yeast cell two forms of the

glycyl tRNA synthetase co-exist, one being sulfide-sensitive and the other equivalent to the sulfide-treated form. It has also been demonstrated that the natural activator of yeast can vary in amount independently of the amount of enzyme.

II. Evolution. Experiments with several uracil derivatives, substituted with alkyl groups in the 5' position, have indicated that 5-isobutyluracil has a greater affinity for a leucine-containing peptide than 5-phenyluracil, 5-benzyluracil, or 5-isopropyluracil. The isobutyl group of leucine is therefore indicated to interact with some degree of specificity with a like group on uracil; a like for like attraction may thus be a basic principle of primitive coding as outlined in our theory, described earlier.

Progress has also been made in the chemical steps required to incorporate unique uracil derivatives into oligonucleotides for the testing of primitive coding capability.

Significance to Biomedical Research and the Program of the Institute:

I. Protein Synthesis. Elucidation of the nature of the enzyme's activation mechanism would open to similar study the regulation of many other of the more complex anabolic reactions of the cell, and reveal basic processes in the control of growth.

II. Evolution. It is hoped that a plausible theory for the origin of living organisms, supported by observation and experiment, will throw light on the nature of fundamental chemical interactions in present-day cells, and that this will be useful in enlarging our understanding and control of disease processes.

Proposed Course:

I. Protein Synthesis. Studies will continue on the activation mechanism of aminoacyl tRNA synthetases.

II. Evolution. Experimental work is being pursued with a view to determining how the first proteins and nucleic acids arose in nature, and how the genetic code originated.

Honors and Awards: None

Publications:

Black, S.: A theory on the origin of life. In Meister, A. (ed.): *Advances in Enzymology and Related Areas of Molecular Biology*. New York, New York, John Wiley & Sons, 1973, Vol. 38, pp. 193-234.

Serial No. NIAMDD-LBP-2
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Chemotherapy of Mouse Leprosy

Previous Serial Number: Same

Principal Investigator: Dr. Yao Teh Chang

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	2.1
Professional:	1.0
Other:	1.1

Project Description:

Objectives:

The evaluation of therapeutic effectiveness of drugs in mouse leprosy. The tissue cultures of intracellular parasites.

Methods Employed:

Chemotherapeutic studies were performed in mice infected intraperitoneally with *Mycobacterium lepraemurium*. Further attempts to cultivate *M. leprae* were made using cultures of mouse macrophages maintained in medium containing very high concentration of serum. Cytochemical and cinemicrographic studies of granulocytic colonies and macrophages were performed in cultures in Leighton tubes and Sight chambers. Grid organ cultures were also used to harvest macrophages of various tissues of mice.

Major Findings:

I. Long-term cultivation of macrophages was achieved from the following sources of the mouse: spleen, fetal liver, lungs, subcutaneous tissue, alveolar washings, and blood. The following technique was also used to grow the macrophages of other tissues. Grid organ cultures were made first. Crops of macrophages were selectively harvested among various types of cells on the cover slips placed underneath the grid culture. Good growth of macrophages were obtained from the thymus, lymph nodes, and heart muscles. Motion pictures of all these macrophages were taken.

II. Further studies on the new chemotherapeutic test for murine leprosy, *i.e.*, using the survival time of animals as the criterion for drug evaluation, revealed that this test was sensitive even to the small doses of drugs (isoniazid, B663 and B1912) administered for a short period after the infection had fully developed. This test seemed to be the most satisfactory model for experimental chemotherapy of murine leprosy.

III. A motion picture of macrophages was prepared and shown at the 2nd Workshop Conference Hoechst on "Activation of Macrophages" at Schloss Reisenburg, Germany. Two types of macrophages were included: the small macrophages and the very large "megamacrophages." Many properties of macrophages were shown, *i.e.*, the pseudopodal formation, ruffling, undulating movement, phagocytosis of small and large dead cells, garbage removal, cell division and cell fusion, and two unusual phenomena of macrophages: (1) the successive formation and burst of large fluid droplets within macrophages and (2) the worm-like configuration of macrophages. This film was also borrowed and shown at the Oxford University, London, by Dr. Colin McDougall, and at the National Institute for Medical Research, London, by Dr. R. J. W. Rees.

IV. The Mickle homogenizer has been routinely used in the mouse foot pad-*M. leprae* experiments for preparation of bacillary homogenates. One major disadvantage of Mickle is the bacterial clumps which remained in the homogenates. We found a simple way to break the clumps by using the Tenbrock glass grinder. Tissue pieces were pressed against the side wall of the grinder instead of its bottom. Pressure can be applied to all the pieces of tissue with little clumps remaining in the homogenates. This method has been found satisfactory in the preparation of homogenates of foot pads infected with both the *M. leprae* and *M. lepraemurium* in the past decade in our laboratory.

Significance to Biomedical Research and the Program of the Institute:

Methods for the cultivation of mouse macrophages other than the peritoneal exudate cells have been lacking. The success in the growth of macrophages obtained from various sites and tissues, including the blood and alveolar cells, offers an opportunity to study the properties of these cells in different tissues and systems. Moreover, the long-living macrophages do multiply in the cultures, contrary to the general belief that macrophages can only be maintained alive in cultures for a limited duration of time.

The simple method in elimination of the bacterial clumps in the foot pad homogenates is very useful, because the Mickle homogenizer which is currently used as the standard homogenizer to prepare the bacterial suspension, reveals too many bacterial clumps in the homogenates. A method to eliminate the clumps is of paramount importance.

Proposed Course:

Studies will be continued on the cultivation of macrophages obtained from various sites and organs of animals, including humans; on the growth of *M. leprae* in macrophage cultures; on cytochemical and cinemicrographic studies of host-parasite relationships; on *in vitro* granulocytopoiesis; and on drug screening in murine leprosy.

Honors and Awards:

I was invited by the Farbwerke Hoechst AG to present a paper at the Workshop on Activation of Macrophages, at Schloss Reisenburg, Germany, in October 1973.

Publications:

Chang, Y. T., and Andersen, R. N.: Studies on the long-term growth of macrophages obtained from various sources (with a presentation of a motion picture). *Excerpta Medica*, in press.

Serial No. NIAMDD-LBP-3
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies in Traumatic Shock and Cellular Immunity

Previous Serial Number: Same

Principal Investigator: Dr. Kehl Markley

Other Investigators: Dr. Sanford M. Rosenthal and Mrs. Elizabeth T. Smallman

Cooperating Units: None

Man Years

Total:	3.1
Professional:	2.3
Others:	0.8

Project Description:

Objectives:

I. To reinvestigate the role of histamine in traumatic shock.

II. To find new methods to prolong skin allograft survival time.

III. To study the influence of age and hormones on local swelling of the mouse tail and ear produced by decreased local atmospheric pressure.

Methods Employed:

I. Burn trauma was produced by immersing mice to the axilla (2/3 body surface area) into water at 70° C for 8 seconds; tourniquet trauma by the application of bilateral hind leg tourniquets for 90 minutes; and endotoxin shock by the intraperitoneal injection of 0.5 mg *E. coli* lipopolysaccharide. Serum and urine histamine was determined by the conversion of histamine and 5-adenosylmethionine [^{14}C]methyl and histamine-N-methyl transferase to [^{14}C]-methyl histamine. Drugs used include histamine diphosphate, serotonin, ATP, compound 48/80, and the histamine inhibitors, benadryl, burimamide, and metiamide.

II. Donor C_{57}BL/6N (H-2^b) skin was incubated for 7-14 days in Eagles' medium with 10% calf serum at 37° C in 5% CO_2-95% air as described by Summerlin. Afterwards skin was placed on C_3H/HeN (H-2^k) recipients and graft

survival time measured.

III. Decreased local atmospheric pressure was applied to the mouse tail as described previously to produce local edema. A similar method was developed to study swelling of the mouse ear. NIH female mice of various age groups were utilized and drugs were injected intraperitoneally prior to subjecting the mice to the reduced pressure.

Major Findings:

I. Serum histamine rose from 50-90% over control values after thermal or tourniquet trauma, but not after endotoxin shock. Pretreatment with histamine produced high levels of serum histamine, but a markedly decreased burn mortality. The administration of four doses of compound 48/80 before burning produced a rise in serum histamine, but also a significant decrease of burn shock mortality. Therefore, there was no good correlation of high mortality with increased serum histamine. Although the administration of antagonists of either the H_1- or H_2-histamine receptors singly had no effect on burn mortality, pretreatment with a combination of the two (benadryl and burimamide) increased shock mortality as early as 2 hours postburn. These results suggest that histamine is not a cause of burn shock mortality but, on the contrary, exerts a compensatory beneficial effect after thermal trauma.

In tourniquet and endotoxin shock, however, histamine produced deleterious effects as a consequence of its action on H_1-histamine receptors.

II. The widely publicized results of Summerlin on the permanent acceptance of skin allografts from different H-2 classes of animals after incubation of the donor grafts in tissue culture medium for 7-14 days were not corroborated.

III. Measurement of the effect of age on transvascular fluid movement by the application of a mild vacuum to the ear or tail of mice demonstrated a large decrease of swelling which correlated with growth. This result was not due to changes in subcutaneous pressures or compliance.

Significance to Biomedical Research and the Program of the Institute:

These studies aid in the understanding of the mechanism of death due to trauma and of transvascular fluid movement.

Proposed Course:

I. To study the function of lymphocytes in cellular immunity, especially after trauma.

II. To search for chemical mediators produced by trauma which alter the immune response.

III. To study the effect of vasoactive hormones on transvascular fluid movement.

Honors and Awards: Dr. Sanford M. Rosenthal was awarded the Harvey S. Allen Distinguished Service Prize from the American Burn Association in 1973.

Publications:

Markley, K., and Thornton, S. W.: Skin graft prolongation caused by changes in grafting procedure. *Transplantation* 16: 80-82, 1973.

Rosenthal, S. M.: The influence of proteolytic enzyme inhibitors on traumatic swelling. *J. Trauma* 13: 548-553, 1973.

Serial No. NIAMDD-LBP-4
1. Biochemical Pharmacology
2. Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Biology of Complex Carbohydrates

Previous Serial Number: Same

Principal Investigator: Dr. Victor Ginsburg

Other Investigators: Drs. David A. Zopf and Alan M. Jeffrey

Cooperating Units: None

Man Years:

Total:	3.7
Professional:	3.0
Others:	0.7

Project Description:

Objectives:

To investigate the role of complex carbohydrates in cell biology.

Methods Employed:

Usual biochemical techniques.

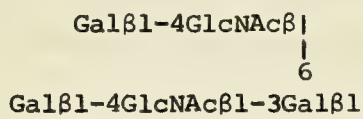
Major Findings:

Specific sugar sequences found in the free oligosaccharides of human milk also occur in glycoproteins and glycolipids of cell surfaces and are important determinants of immunologic specificity. Some sequences predominate in certain human tumors as contrasted to corresponding normal tissue where they are either absent or present at low levels. Antisera directed against these sequences should be useful diagnostically as evidenced by the importance of the carbohydrate "carcino-embryonic" antigen described by Gold and Freedman for the detection of human colonic carcinomas. We are preparing antibodies against pure milk oligosaccharides for possible use in the detection of "tumor antigens" and for studies on changes in cell surface sugars during malignant transformation.

Oligosaccharides isolated from milk were coupled to polylysine (MW 70,000) by a modification of the mixed-anhydride procedure of Arakatsu, Ashwell, and

Kabat. From 20 to 80 moles of oligosaccharide were coupled per mole of polylysine. The products were effective precipitating antigens as evidenced by tests with antisera of known specificity. For example, the lacto-N-fucopentaose II conjugate reacted specifically with antibodies directed against the human "Lewis a" blood group determinant, while the lacto-N-difucohexaose I conjugate reacted specifically with antibodies directed against the human "Lewis b" blood group determinant.

The conjugates were used to test the specificity of a human anti-I cold agglutinin (called "Ma") obtained from a patient with an autoimmune hemolytic anemia. The agglutinin reacted specifically with the lacto-N-neohexaose conjugate. Lacto-N-neohexaose (LNnH), a hexasaccharide isolated and characterized in this laboratory, has the following structure:



As LNnH has recently been found in glycolipids of human tissue, it is possible that this sugar is the natural red cell target of the agglutinin *in vivo*. LNnH contains the sequence of sugars Galβ1-4GlcNAcβ1-6Gal previously shown by Kabat *et al.* to inhibit the anti-I of "Ma" serum. Another purified human anti-I cold agglutinin (called "Step") did not react with the LNnH conjugate or any of the other conjugates.

Affinity columns are useful for the purification of antibodies. However, available methods are unsatisfactory for the preparation of affinity columns where the ligands are acid-labile oligosaccharides available only in limited quantities, either because of low yields or because of the drastic conditions required for reaction. For this reason, the following procedure was developed, using the bifunctional reagent ω-(p-aminophenyl)-alkylamine. Alkylamines react with sugars to form easily hydrolyzed n-alkylglycosylamines which can be reduced to stable 2° amines. Arylamines, in contrast, do not react in the absence of a catalyst but couple more readily at neutral pH with CNBr-activated Sepharose. For an example of coupling, the pentasaccharide, lacto-N-fucopentaose I (LNF I) (100 μmoles), was reacted with β-(aminophenyl)-ethylamine (500 μl), reduced with $NaBH_4$ in ethanol, and excess amine removed by column chromatography on G-10 Sephadex (if excess amine is not used, in addition to the desired 2° amine, 3° amine is also obtained by disubstitution together with some reduced sugar). Substituted LNF I was obtained in quantitative yield based on the uv adsorption of the product and fucose (the distal sugar from the reducing terminus of LNF I) estimations. The material was then coupled quantitatively to CNBr-activated Sepharose (6 μmoles of derivatized sugar/ml of gel) in sodium bicarbonate buffer as measured by the uv adsorption of the liquid phase. Excess reactive sites were blocked with ethanolamine.

Significance to Biomedical Research and the Program of the Institute:

These studies may contribute to an understanding of cell-cell interactions which are important to biological phenomena such as graft rejection, autoimmune diseases, and carcinogenesis.

Proposed Course:

We will continue to try to elucidate the carbohydrates on cell surfaces that are responsible for their specificity.

Honors and Awards: None

Publications:

Zopf, D. A., and Ginsburg, V.: Carbohydrate antigens of cell surfaces. In Lee, E. Y. C. (ed.): *Biology and Chemistry of Eucaryotic Cell Surfaces.* New York, New York, Academic Press, 1974, in press.

Serial No. NIAMDD-LBP-5
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Estimation, Metabolism, and Function of Amines

Previous Serial Number: Same

Principal Investigators: Drs. Herbert Tabor and Celia White Tabor

Other Investigator: Dr. Edmund W. Hafner

Cooperating Units: None

Man Years:

Total:	3.8
Professional:	2.3
Other:	1.5

Project Description:

Objectives:

The importance of the various polyamines is shown by their wide distribution in viruses, bacteria, plant cells, and animal cells. Studies are being conducted to elucidate their metabolism and function.

Methods Employed:

The amines and their derivatives are studied by isotopic labeling and special chromatographic techniques. Standard enzymological techniques have been used to purify further the enzymes that synthesize and degrade spermine, spermidine, and their derivatives. Chemical synthetic procedures have been utilized to prepare derivatives for identification of enzymatic products. Uptake of amines was studied by counting the residual radioactivity in washed pellets on Millipore filters. Gel electrophoresis techniques have been utilized to study the ribosome pattern of cells. Amino acid analyses were carried out in an automatic amino acid analyzer. Fluorometric analyses have been utilized.

An amino acid analyzer has been utilized for the development of a new, automated, sensitive, and quantitative method for the analysis of amines and amine derivatives.

The metabolism of glutathione in *Escherichia coli* is being studied both with isotopic techniques and by the development of an automated system of analysis, using an amino acid analyzer.

Genetic techniques are being used to select organisms with genetic defects in the synthesis of the polyamines.

Major Findings:

The work in this area has largely centered around the compound, glutathionylspermidine, which we have isolated from *E. coli* at the beginning of stationary phase. The importance of this compound is indicated by quantitative assays showing that all of the bacterial spermidine and half of the intracellular glutathione are found in this form under these growth conditions.

Methods have been developed for the convenient isolation of relatively large amounts of glutathionylspermidine from *E. coli* and for its determination in the amino acid analyzer. Additional structural studies have shown that the glutathione is linked to the N^1-position of the spermidine.

Other related studies have been concerned with the substrate specificity and products of the *Serratia* spermidine dehydrogenase and on gas chromatographic methods for the polyamines. Several modifications have also been introduced into our automated method for the determination of amines in order to separate the amines from their methylated derivatives.

Studies have been started on the content of the polyamine synthesizing enzymes in various mutants of *E. coli*.

Significance to Biomedical Research and the Program of the Institute:

Recent work has indicated that the polyamines may play an important role in permeability of organisms and in the stability of nucleic acids inside cells. Studies by Pardee and Maas suggest that the amines may also play an important role in the division of *E. coli*. Polyamines have been implicated in control of RNA, DNA, and protein syntheses.

Some of the polyamines have also been found to act as growth factors for bacteria, plants, and certain mammalian cell lines in culture, as well as in the response of mammalian tissues to hormones and regeneration. Our studies of the amine content and metabolism in bacteria and mammalian cells under various growth conditions will contribute to our understanding of the functions of these ubiquitous compounds.

Our studies on the propylamine transferase enzyme may lead to elucidation of the mechanism of the formation of the C-N bond in bacteria and the role of the high-energy sulfonium ion in this reaction.

Proposed Course:

Further studies on the metabolism and function of the biologically important polyamines. We hope to learn the normal physiological function

of these amines in the cell and to explain such pathological effects as the production of renal damage by spermine.

Honors and Awards: None

Publications:

Tabor, H., Tabor, C. W., and Irreverre, F.: Quantitative determination of aliphatic diamines and polyamines by an automated liquid chromatography procedure. *Anal. Biochem.* 55: 457-467, 1973.

Serial No. NIAMDD-LBP-6
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Enzymatic Studies of Nucleic Acid Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. Nancy G. Nossal

Other Investigator: Dr. Frances D. Gillin

Cooperating Units: Dr. Michael Gottesman, Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases

Man Years:

Total:	2.2
Professional:	1.7
Other:	0.5

Project Description:

Objectives:

To determine the characteristics and functions of enzymes which are involved in the synthesis, modification, and degradation of DNA.

Methods Employed:

Standard biochemical techniques were employed for the preparation of polynucleotide substrates and enzymes. Extensive use has been made of amber mutants of T4 bacteriophage which are unable to make specific early enzymes in nonpermissive hosts. Procedures have been developed for the large scale preparation of these amber mutants and for the production of kilogram quantities of infected bacterial cells.

Major Findings:

I. DNA Synthesis by the T4 Polymerase in the Presence of the Gene 32 DNA Unwinding Protein. The DNA polymerase induced by T4 bacteriophage is one of several proteins which are absolutely required for the replication of the phage DNA *in vivo*. This polymerase has both polymerase and 3' to 5' exonuclease activities in a single polypeptide of MW 110,000. Genetic and biochemical

studies with mutants in the structural gene for this enzyme suggest that this polymerase is involved in the replication of the linear duplex DNA of the virus. *In vitro*, however, the enzyme is unable to copy double stranded DNA, and its copying of single stranded DNA is inefficient compared with its rate *in vivo*, in part because its exonuclease activity carries out extensive hydrolysis of newly incorporated residues. Thus it appears likely that the addition of other phage induced proteins will be necessary in order to reconstruct a system capable of copying native duplex DNA *in vitro*.

DNA synthesis with a duplex template requires the displacement of the DNA strand which is annealed to the strand serving as the template. The failure of T4 DNA polymerase to copy such a template appears to result from the inability of the enzyme to displace this strand. We have previously shown that the phage polymerase will use a nicked duplex template in the presence of the gene 32 DNA unwinding protein, which would be expected to promote the strand displacement. This protein, which is encoded by the phage gene 32, has been shown by Alberts and his collaborators to be required in high concentrations for T4 DNA synthesis *in vivo* and to promote DNA strand unwinding *in vitro*.

We have now characterized the products of the copying of a small nicked duplex DNA (T7 DNA) by the T4 DNA polymerase in the presence of the DNA unwinding protein. The major product is a rapidly renaturable DNA with the same base composition as the template. At least some of the product is covalently attached to the DNA serving as the template-primer. The renaturability of this product was shown by its banding position after equilibrium centrifugation in neutral CsCl and its resistance after heat denaturation to the single strand specific S_1 nuclease. In electron micrographs made in collaboration with Dr. Michael Gottesman, this product contained branches before denaturation, and appeared as highly branched or hairpin molecules after heating and rapid cooling of the DNA. These renaturable hairpin molecules would result if at some point during the reaction the polymerase stopped copying the template strand and began instead to copy either the strand serving as the primer or the displaced strand. Presumably this is prevented *in vivo* by one or more of the other proteins required for T4 DNA replication.

The alternating polymer poly[d(A-T)] is also formed to a variable extent during the later stages of the T7 DNA primed reaction with the T4 DNA polymerase and the 32 protein. It probably results from the creation of a poly-[d(A-T)] template by continuous strand slippage after the copying of a region of T7 DNA with a short stretch of alternating A and T residues. This strand slippage may be facilitated by the 32 protein, since this unwinding protein greatly stimulates both the rate and extent of the copying of a poly[d(A-T)] template by the T4 DNA polymerase.

II. T4 Gene 44 and Gene 62 Proteins. The proteins encoded by T4 phage genes 44 and 62 are also required for T4 DNA synthesis *in vivo*, and have been shown by Barry and Alberts to exist as complex. We have extensively purified this protein using as an assay the stimulation of DNA synthesis in a lysate made from *Escherichia coli* which has been infected with a gene 44 mutant of T4 phage. The purified protein does not have DNA polymerase, nuclease, or

deoxynucleoside triphosphatase activity. We are presently trying to find conditions under which the gene 44-62 protein will stimulate DNA synthesis catalyzed by the T4 polymerase and the DNA unwinding protein.

III. Control of Mutation Rates by the T4 DNA Polymerase. The T4 DNA polymerase offers a good system in which to study factors controlling the fidelity of DNA replication. Some mutations in the structural gene for this enzyme produce a dual phenotype: first DNA synthesis *in vivo* is temperature sensitive, and second, at permissive temperatures the rate of mutations produced in other phage genes is either increased (mutator phenotype) or decreased (antimutator phenotype). Since the enzyme has both polymerase and 3' to 5' exonuclease activities, changes in the accuracy of DNA replication with the mutant enzymes could result from differences in the ability of the enzymes to discriminate between correct and incorrect nucleotides during incorporation, and from differences in the efficiency of the removal of noncomplementary nucleotides by the exonuclease activities of the mutant enzymes.

We have compared the purified DNA polymerase induced by the wild type T4 phage with that induced by a strong mutator (*ts* L88) and a strong antimutator (*ts* CB120). We have used the homopolymers poly dA and poly dT as a model system for studying the frequency of *in vitro* copying errors relative to correct DNA synthesis. In this system the mutator enzyme catalyzes the utilization of both noncomplementary nucleotides (dGTP and dCTP) more frequently than the wild type enzyme, while the antimutator enzyme incorporates each of the wrong nucleotides less frequently than the wild type enzyme. With all three enzymes, the utilization of the noncomplementary nucleotide must be measured by the DNA dependent conversion of deoxynucleoside triphosphate into monophosphate. There is no measurable stable incorporation of the wrong nucleotides, apparently because they are so efficiently removed by the exonuclease activity of each enzyme. Thus with this template the three enzymes appear to differ in their frequencies of incorporation of noncomplementary nucleotides rather than in the rates of removal of erroneous nucleotides by their nuclease activities. The antimutator enzyme consistently hydrolyzed a larger fraction of newly incorporated complementary nucleotides than the wild type or mutator enzymes. Studies of the effect of increasing concentrations of complementary nucleotides on the utilization of incorrect nucleotides have not yet revealed any qualitative differences in the types of errors made by these three enzymes. Experiments are in progress to measure apparent binding constants for each of the nucleotides in the presence of complementary and noncomplementary templates.

The three polymerases also differ in their interaction with the gene 32 DNA unwinding protein *in vitro*. The 32 protein greatly stimulates both the rate and the extent of the copying of the alternating polymer poly[d(A-T)] by the wild type polymerase under conditions which suggest that it acts by unwinding regions of intrastrand hydrogen bonding in the polymer serving as the template which interfered with the movement of the polymerase. In contrast, the 32 protein stimulates the *ts* L88 mutator polymerase only about 2-fold under the same conditions. The *ts* CB120 antimutator polymerase has no detectable stable incorporation of nucleotides with poly[d(A-T)] in the absence of the 32 protein and only a very low rate of the DNA dependent

conversion of deoxynucleoside triphosphate into monophosphate. In the presence of the DNA unwinding protein the rates of both stable incorporation and deoxynucleoside monophosphate production are so greatly stimulated that the sum of these reactions with the antimutator enzyme exceeds that with the wild type enzyme. Thus in this system the *ts* CB120 mutant enzyme appears to be defective in its ability to copy a template with intrastrand secondary structure, rather than in its ability to catalyze the incorporation of new nucleotides *per se*.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of enzymes controlling DNA synthesis and degradation is important to an understanding of the control of DNA replication and transcription in both normal and viral infected cells.

Proposed Course:

Our long range goal is to reconstruct a system of bacteriophage T4 proteins which is capable of synthesis of duplex T4 DNA. We will try to determine the function of the gene 44-62 protein. Using an assay analogous to that used for the 44-62 protein, we will attempt to purify and characterize the products of other phage genes known to be required for T4 DNA replication. We will continue our study of the control of mutation rates by the T4 DNA polymerase.

Honors and Awards: None

Publications:

Hershfield, M. S., and Nossal, N. G.: *In vitro* characterization of a mutator T4 DNA polymerase. *Genetics* 73: 131-136, 1973.

Nossal, N. G.: DNA polymerase from wild type and mutant T4 bacteriophage. In Wickner, R. B. (ed.): *DNA Replication and Biosynthesis, Methods in Molecular Biology*. New York, New York, Marcel Dekker, Inc., 1974, Vol. 9, in press.

Nossal, N. G.: DNA synthesis on a double-stranded DNA template by the T4 bacteriophage DNA polymerase and the T4 gene 32 DNA unwinding protein. *J. Biol. Chem.* 249, 1974, in press.

Nossal, N. G., and Hershfield, M. S.: Exonuclease activity of wild type and mutant T4 DNA polymerases. Hydrolysis during DNA synthesis *in vitro*. In Wells, R. D., and Inman, R. B. (eds.): *DNA Synthesis in Vitro*. Baltimore, Maryland, University Park Press, 1973, pp. 47-62.

Serial No. NIAMDD-LBP-7
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Protein Synthesis in *Escherichia coli*

Previous Serial Number: Same

Principal Investigator: Dr. Anthony V. Furano

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.1
Professional:	1.0
Others:	0.1

Project Description:

Objectives:

To study the mechanism and control of protein synthesis. This laboratory is also involved in studying the processes and effects of protein modification (*e.g.*, methylation) in collaboration with Dr. Michael Klagsbrun (Department of Surgery, Children's Hospital Medical Center, Boston, Massachusetts).

Methods Employed:

This laboratory has purified the peptide chain elongation factors present in *E. coli* and thereby devised methodology to measure directly the levels of these factors in these bacteria under varying conditions of growth. Studies are also being carried out on the elongation factors *in vitro*.

Protein Modification (in collaboration with Dr. M. Klagsbrun). We are identifying the types of methylated amino acids present in the various subcellular fractions of *E. coli* and mammalian cells.

Major Findings:

I. Elongation Factors. Work from other laboratories has shown that three elongation factors are obtainable from extracts of *E. coli* and are involved in ribosomal dependent peptide chain elongation *in vitro*: Tu, Ts, and G. (Tu and Ts are usually obtained as a complex, Tu-Ts, called T.) Both T (*i.e.*, Tu-Ts) and G are required for polypeptide synthesis. However, earlier work from this laboratory and others demonstrated the existence of yet another elongation

factor (or another form of the elongation factors) which had the property of promoting peptide chain elongation by itself, *i.e.*, it did not require the addition of T or G and was called (G+T). Work from other laboratories had shown that this factor was not separable into T and G by methods that were shown to resolve these two activities, and (G+T) could not be produced by mixing previously separated T and G. Furthermore, work from this laboratory suggested that although (G+T) was stabilized by the proteolytic inhibitor, phenylmethylsulfonyl fluoride, and could be partially purified in its presence, we did note that the inhibitor did not completely prevent the loss of (G+T) especially during chromatography on DEAE-Sephadex. It thus seemed possible that the failure of others to observe (G+T) could be due to the instability of this factor on DEAE-Sephadex, and accordingly we sent out to purify the elongation factors without using this chromatographic medium. In the course of this work, as well as in our earlier work, it was noted that material with T activity and G activity behaved quite heterogeneously during fractionation. Therefore, methods were developed to identify which of the protein bands seen on disc gel electropherograms correspond to Tu, Ts, and G, and thereby it was possible to monitor all of the fractions obtained during the purification of these factors for their content of the various elongation factors. In this way a comprehensive description of the state and nature and relative amounts of the elongation factors present in ribosome-free extracts of *E. coli* was made.

Before presenting these results it is important to point out that polypeptide synthesis was used as the functional assay for the elongation factors, and it was found quite early that Tu alone as well as Tu-Ts (T) could complement G in carrying out peptide chain elongation *in vitro*; *i.e.*, Ts is not absolutely required, at least *in vitro*. (This dispensability of Ts has also been shown by others.) At any rate for polypeptide synthesis at least, fractions exhibiting at least "T activity" can contain either Tu alone or Tu-Ts (*i.e.*, T). The salient features of the results then are as follows.

(1) The material with the activity and chromatographic properties of (G+T) is either a very weak complex of G and Tu or an admixture of Tu and G resulting from their fortuitous cochromatography on various media.

(2) Tu exists in the cell as either free Tu, as bound to Ts (*i.e.*, as T), or as loosely aggregated with G or some other cellular component(s). To be certain that three three Tu's were in fact the same polypeptide, cells were labeled with [^{14}C]arginine and peptide maps were prepared from the tryptic digests of free Tu, the Tu bound to Ts, and aggregated Tu. The peptide maps of all three were identical and corresponded well with the number of arginines determined by amino acid analysis.

(3) In the course of this work, methods were developed for determining directly the amounts of radioactive Tu and G in cell extracts, and it was found that for every mole of ribosomes there is about 1 mole of G (in agreement with what others have found) and about 9 moles of Tu. This is substantially more (in fact, 9 times more) Tu than was previously thought to be present in cells, and the bulk of the Tu appears to be free Tu, *i.e.*, not bound to Ts in the form of T or aggregated to other molecules.

II. Methylamino Acids. A comparative study of the methylated amino acids in culture 3T3 mouse cells and *E. coli* was made. Cells were labeled with [^{14}C]-methylmethionine and then fractionated into crude pellet (which consists mainly of nuclei with the mammalian cells and membranes with the bacterial cells), ribosome-free cytoplasm, small ribosomal subunit (40S for the mouse cells and 30S for the bacteria), and large ribosomal subunit (60S for the mouse cells and 50S for the bacteria). Portions of these fractions were hydrolyzed and their content of methylamino acids were analyzed by 2-dimensional thin layer chromatography. As had been shown for methylated nucleic acid bases, the bacterial extracts contained smaller amounts and fewer kinds of methylated amino acids than did the mammalian extracts. The ribosome-free cytoplasm and pellet obtained from *E. coli* revealed only small amounts of N,N'-dimethylarginine.

The 30S subunit also contained N,N'-dimethylarginine and three as yet unidentified spots, one of which might be a methylated histidine. The 50S subunit showed a more complex pattern which except for N,N'-dimethylarginine and one of the unidentified spots was distinct from that shown by the 30S subunit. The 50S subunit contained dimethyllysine, methylysine, a spot tentatively identified as trimethyllysine, and an unidentified spot, in addition to the unidentified one it shares with the 30S subunit.

The patterns obtained from the 3T3 cells were more complex and appeared to contain larger amounts of the methylated amino acids. The nuclei-containing pellet contained only those methylated amino acids that have been described for histones and in fact showed essentially the same pattern as that obtained from the hydrolysate of [^{14}C]methyl-labeled histone prepared from 3T3 cells; Mono-, di-, and probably trimethyllysine, N,N-dimethylarginine (or N-methylarginine) were the predominant amino acids identified. The ribosome-free cytoplasm had an essentially similar pattern except that it lacked methyllysine and contained the N,N'-dimethylarginine that was not present in the pellet. The 40S subunit contained N,N-dimethylarginine (or N-methylarginine) as its most prominent methylated amino acid and also a spot which as in the smaller subunit of *E. coli* was tentatively identified as a methylhistidine. The presence of these two spots distinguished the 40S from the 60S which contained small amounts of mono-, di-, and probably trimethyllysine, N,N'-dimethylarginine, and an unidentified spot which was common to the 30S and 50S subunits of bacteria.

In summary, the cytoplasm and crude pellet of *E. coli* (which has been shown to lack histones) contain only small amounts of methylamino acids. Except for an unidentified spot on the 30S and a methylarginine (N,N-dimethyl or N-methyl) on the 40S, the small ribosomal subunits of bacteria and mouse are similar in pattern, if not amount. Except for one unidentified spot in the bacterial 50S subunit, the large ribosomal subunits are similar in pattern. The mouse ribosome-free cytoplasm contains large amounts and more types of methylated amino acids than the ribosome-free cytoplasm of bacteria.

Significance to Biomedical Research and the Program of the Institute:

Knowledge on the mechanism of protein synthesis and the processes and effects of protein modification (*e.g.*, methylation) is essential to understanding cellular metabolism and growth.

Proposed Course:

This work is currently in progress and will be continued and extended.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LBP-8
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Methylation of RNA and Protein in Prokaryote and Eukaryote Organisms

Previous Serial Number: Same

Principal Investigator: Dr. Michael Klagsbrun

Other Investigators: None

Cooperating Unit: Dr. Philip M. Farrell, Pediatric Metabolism Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases

Man Years:

Total:	0.0
Professional:	0.0
Other:	0.0

Project Description:

This project is terminated.

Publications:

Klagsbrun, M., and Farrell, P. M.: The methylation of RNA in fibroblasts of normal volunteers and patients with cystic fibrosis. In Mangos, J. A., and Talamo, R. C. (eds.): *Fundamental Problems of Cystic Fibrosis and Related Diseases*. Miami, Florida, Symposia Specialists, 1973, pp. 53-61.

Klagsbrun, M., and Farrell, P. M.: The methylation of transfer and ribosomal ribonucleic acid in human fibroblasts: normal methylation of ribonucleic acid in cystic fibrosis. *Pediat. Res.* 8: 205-211, 1974.

Serial No. NIAMDD-LBP-9
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Chemical and Physiological Properties of the Surface of *Escherichia coli*

Previous Serial Number: Same

Principal Investigator: Dr. Loretta Leive

Other Investigator: Dr. Pinakilal Bhattacharyya

Cooperating Units: None

Man Years:

Total:	2.9
Professional:	2.1
Other:	0.7

Project Description:

Objectives:

To study the assembly, structure, and physiology of the outer membrane of *E. coli*.

Methods Employed:

Usual biochemical methods, including isolation and purification of cell surface components by extraction, ultracentrifugation, chromatography, and selective enzymatic degradation, determination of the size and shape of these fractions by biophysical measurements of density, viscosity, and ultracentrifugal properties, identification and quantitation of sugars by colorimetric and enzymatic procedures, and use of labeled compounds to specifically label cell fractions *in vivo*.

Major Findings:

Structure and Assembly of Escherichia coli Membranes. Previous work in this laboratory using density labeling of lipopolysaccharide (LPS) indicated that there were about 10 to 30 specific insertion points in the outer membrane, into which newly-synthesized LPS was translocated from the inner membrane, and from whence it spread to the remainder of the outer membrane. Work in another laboratory has now confirmed this finding by another technique, electron

microscopic visualization of ferritin-labeled antibody to newly-synthesized LPS. These experiments seem to suggest that the newly-synthesized LPS might be emerging into the outer membrane through bridges between the two membranes. The outer membrane above such bridges is known as the site of adsorption of several phages, including phage BF23. Since recently the receptor for this phage has been found to be the same as the receptor for cyanocobalamin we decided to see whether the insertion points for LPS, isolated by our technique, contained higher concentrations of cyanocobalamin receptor, as measured by extent of binding of radioactive cyanocobalamin. Experiments to date indicate no higher binding of cyanocobalamin by the insertion points as compared to the remainder of the outer membrane, although the finding of another laboratory that receptor is present only in the outer membrane was confirmed.

When the density gradient technique of separating insertion points from the remainder of the outer membrane was used to prepare large quantities of each for analysis, it became apparent that the technique did not provide sufficient resolution for complete separation of the two types of membrane fragments. For this reason another method of separation was sought. LPS is a good antigen and antisera prepared by LPS injection are specific for the carbohydrate terminus of the molecule. When galactose is added to a UDP-galactose epimerase-less mutant, it instantly changes from production of one antigenic type (1) of LPS to another (2). By adsorbing sera from rabbits injected with type 2 LPS with either whole cells or isolated LPS of type 1, it is possible to prepare antibody highly specific for only type 2 LPS. Such antibodies were prepared and coupled to Sepharose, and used for affinity chromatography. Labeled type 2 LPS was shown to adsorb to the column and to be specifically eluted with non-radioactive type 1 LPS or with carbohydrate haptene produced from such LPS. In preliminary experiments, outer membranes prepared from the mutant have been shown to adsorb to the column if, and only if, the mutant is grown in the presence of galactose (producing type 2 antibody) and not if it is not grown in the absence of galactose (producing type 1 antibody). When adsorbed, such membrane fragments can be eluted by type 2 LPS or haptene. When perfected the technique will be used for fractionation of outer membrane fragments from cells that have just begun production of type 2 LPS, in an effort to isolate outer membrane insertion point fragments, which are enriched for new LPS.

Significance to Biomedical Research and the Program of the Institute:

Study of the organization of the outer layer of the cell surface will contribute to understanding its structure and its interaction with the environment. Earlier work from this laboratory showed that the outer layer is the barrier to many charged molecules in coliform bacteria, including many drugs, and that this barrier can be reduced by exposing the cells to EDTA, thus making them drug-sensitive. The current studies are aimed at understanding the structural basis for this permeability barrier, and such understanding may have implications for drug therapy.

Furthermore, the very heterogeneous membrane structure suggested by the preceding experiments, in which different areas are specialized for insertion of different molecules that then can move within the membrane to achieve their final location, appears similar to the situation in cells of higher organisms,

in which heterogeneity of carbohydrate-containing macromolecules on the cell surface is thought to play an important role in cell-cell interactions, differentiation, and malignancy. It is hoped that the greater ease of analyzing the outer membrane of *E. coli*, which is simpler than the membranes of higher organisms, will provide information on the mechanisms of assembling and arranging heterogeneous membranes.

Proposed Course:

The specialized regions of LPS entry mentioned above will be isolated, and their composition further analyzed. The rate of diffusion of LPS from these points to the rest of the outer membrane will also be measured. By specifically labeling lipid, protein, and peptidoglycan, the relationship of the insertion points for LPS to the location of newly inserted other components will be measured.

Honors and Awards: None

Publications:

Leive, L. (Ed.): *Bacterial Membranes and Walls*. New York, Marcel Dekker, Inc., 1973, 495 pp.

Serial No. NIAMDD-LBP-10
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Chemistry and Mechanism of Pyridoxal Phosphate Enzymes

Previous Serial Number: Same

Principal Investigator: Dr. Edith Wilson Miles

Other Investigator: Dr. Osao Adachi

Cooperating Units: None

Man Years:

Total:	2.2
Professional:	2.0
Other:	0.2

Project Description:

Objectives:

To gain further understanding of features of enzymes which contribute to their conformation, subunit interaction, and mechanism of action. Pyridoxal phosphate enzymes have been chosen for study because they are important in amino acid metabolism and because pyridoxal phosphate has optical and chemical properties which make it a useful probe of the active site of the enzymes to which it is bound.

Methods Employed:

Enzyme purification procedures were developed to prepare homogeneous β_2 subunit and $\alpha_2\beta_2$ complex of tryptophan synthetase. Crystallization techniques were employed to grow crystals of β_2 subunit and $\alpha_2\beta_2$ complex. Purified enzymes were characterized by disc gel and sodium dodecylsulfate gel electrophoresis, by ultracentrifuge studies, by kinetic studies, and by absorbance and circular dichroism spectra.

Photooxidation studies of the β_2 subunit were carried out in the presence of its cofactor, pyridoxal phosphate, as a photosensitizer. Chemical analyses and amino acid analyses were carried out to identify the residue destroyed by photooxidation as a histidyl residue. Tryptic digestions and fractionation of the resulting peptides by column chromatography and peptide mapping were

carried out to identify the peptide region which contained the modified histidyl residue.

Major Findings:

The β_2 subunit has been purified by a very simple procedure which takes advantage of the different solubilities of the enzyme in the presence and absence of its cofactor, pyridoxal phosphate. The crystalline β_2 subunit has higher activity than any previously reported. The $\alpha_2\beta_2$ complex has also been prepared and crystallized from a synthetic mixture of the α and β_2 subunits and also by a new purification from a strain of *Escherichia coli* which contains both subunits (native complex). Large crystals of the native complex are being grown for x-ray crystallographic studies. The subunit composition and molecular weights of both $\alpha_2\beta_2$ complexes have been determined and found to be the same. Circular dichroism and absorbance spectra of the α and β_2 subunits before and after mixing are being determined in order to elucidate the effects of subunit interaction on the environment of the coenzyme and of aromatic amino acids.

The presence of an essential histidyl residue in the active site of the β_2 subunit has been demonstrated by photooxidation studies. Pyridoxal-P sensitizes the photooxidation of one specific, essential histidyl residue per β monomer with resulting inactivation of the β_2 subunit. This essential histidyl residue has been located in the tryptic peptide of the β_2 subunit which binds pyridoxal-P. This histidyl residue probably serves a catalytic role in removing the α-proton from the substrate, L-serine.

Significance to Biochemical Research and the Program of the Institute:

These studies are clarifying the active site chemistry and mechanism of pyridoxal phosphate enzymes which are important in amino acid metabolism. They also give information on subunit interaction and enzyme regulation in multienzyme systems.

Proposed Course:

These studies will be continued and extended with long range goals of determining chemical and physical features of pyridoxal phosphate enzymes required for specificity, catalytic activity, maintenance of conformation, and protein-protein interaction.

Honors and Awards: None

Publications:

Adachi, O., and Miles, E. W.: A rapid method for preparing crystalline β_2 subunit of tryptophan synthetase of *Escherichia coli* in high yield. *J. Biol. Chem.* 249: in press, 1974.

Miles, E. W.: Evidence that the essential, photosensitive histidyl residue in the β_2 subunit of tryptophan synthetase is in the pyridoxyl peptide. *Biochem. Biophys. Res. Commun.* 57: 849-856, 1974.

Miles, E. W., and Kumagai, H.: Modification of essential histidyl residues of the β_2 subunit of tryptophan synthetase by photooxidation in the presence of pyridoxal 5'-phosphate and L-serine and by diethylpyrocarbonate. *J. Biol. Chem.* 249: 2483-2491, 1974.

Miles, E. W., and McPhie, P.: Evidence for a rate-determining proton abstraction in the serine deaminase reaction of the β_2 subunit of tryptophan synthetase. *J. Biol. Chem.* 249: 2492-2497, 1974.

Serial No. NIAMDD-LBP-11
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Regulation of Cellular Activity by the Action of Pharmacodynamic Agents on Enzymes within the Cell or in Its Membrane

Previous Serial Number: Same

Principal Investigator: Dr. Leonard D. Kohn

Other Investigators: Dr. Jere P. Segrest (July 1973-October 1973), Dr. Ramon L. Tate, and Mr. George Lee (Graduate Student from American University, whose work is accomplished as partial fulfillment of his degree requirements).

Cooperating Units: Dr. Roger J. Winand, Hopital de Baviere, Institut de Medicine, Liege, Belgium
Dr. Charles M. Lapiere, Department of Dermatology, Hopital de Baviere, Institut de Medicine, Liege, Belgium
Dr. Ralph J. Nossal, Physical Sciences Laboratory, Division of Computer Research and Technology, NIH
Dr. H. Ronald Kaback, Roche Institute of Molecular Biology, Nutley, New Jersey
Dr. George R. Martin, Laboratory of Biochemistry, NIDR, NIH
Dr. E. Carwile Leroy, Department of Medicine, Columbia College of Physicians and Surgeons, New York, New York
Dr. Jan Wolff, Clinical Endocrinology Branch, NIAMDD, NIH
Dr. Lois W. Tice, Laboratory of Experimental Pathology, NIAMDD, NIH

Man Years:

Total:	4.2
Professional:	3.7
Other:	0.5

Project Description:

Objectives:

The overall objective is the evaluation of mechanisms through which cellular activities are regulated by the action of pharmacodynamic agents on enzymes within the cell and in its membrane. Specifically, these studies have concerned the mechanism of exophthalmos and Graves' disease, preventive therapeutic measures for these diseases, and hormone receptor function at a membrane

level. These studies have extended to the enzymatic mechanisms of membrane transport in bacterial and mammalian cells; to membrane structure; to enzymatic mechanisms in the biosynthesis of collagen and their relation to human collagen disease states; and to the structure and function of oxidoreductases important to energetic regulation of cell functions.

Methods Employed:

Procedures common to the purification, assay, and chemical modification of enzymes have been coupled with methods evaluating the size, shape, composition, and sequence of macromolecules.

Major Findings:

I. Studies Concerning the Mechanism of Exophthalmos and of Thyrotropin Receptor Function. Our previous work has demonstrated that thyrotropic hormone is exophthalmogenic but that partial pepsin digestion could yield an exophthalmogenic fragment of the thyrotropin molecule which has lost its thyroid stimulating ability. Previous studies concerning the binding of these hormonal factors and their biochemical effects on thyroid and retro-orbital tissue have now led us to suggest that exophthalmos is a two-factor disease requiring the simultaneous presence of thyrotropin (TSH) or a TSH derivative without thyroid stimulating ability, and of an abnormal gamma globulin which is not a long acting thyroid stimulator (LATS) and which can be detected in the serum of patients with exophthalmos. The TSH or the TSH derivative is presumed to be the direct effector; the abnormal gamma globulin is presumed to target this effector by modifying its binding characteristics to retro-orbital receptors.

The requirement for two factors rather than one alone would explain the low incidence of exophthalmos in Graves' disease; the fact that the TSH derivative is without thyroid-stimulating ability would explain the present failure to detect significant TSH activity in the sera of patients with Graves' disease. The TSH derivative could be a precursor or a proteolytic fragment of TSH analogous to the exophthalmogenic factor that was produced by digestion with pepsin, or could be the β subunit, which has 10-30% of the exophthalmogenic activity of this factor. Since the production of a TSH derivative without thyroid-stimulating ability could be a consequence of high circulating concentrations of thyroid hormone, *i.e.*, a consequence of a feedback mechanism reducing the production or increasing the destruction of a native TSH molecule, the absence of the gamma globulin factor in other cases of hyperthyroidism (functioning adenomas, exogenous thyroid) could explain the absence of exophthalmos in these disease conditions.

All the patients with Graves' disease and active exophthalmos who have been tested have a circulating gamma globulin that induces exophthalmos in fish or enhances binding and is not LATS. All the patients with Graves' disease and active exophthalmos who have been tested also have high levels of leukocyte-bound antibodies that interact with antigens from retro-orbital and thyroid tissue as measured by inhibition of leukocyte migration. All patients with Graves' disease and no exophthalmos who have been tested have

no circulating gamma globulin that induces exophthalmos in fish or enhances binding but approximately half of these patients have leukocyte-bound antibodies. Treatment of this last group of patients with Imuran, 1 mg/kg, has eliminated the appearance of exophthalmos during therapy for hyperthyroidism in the entire group of Graves' disease patients who appear without exophthalmos (Winand, R. J., and Mahieu, P., *Lancet*, May 26, 1973, p. 1196).

Studies of TSH receptors in thyroid and retro-orbital tissues have been pursued in an effort to understand the cause for the "abnormal" receptor response inherent in exophthalmos. The binding of TSH to intact and solubilized bovine thyroid receptors has been studied using iodinated TSH preparations. Purified bovine TSH was iodinated with ^{125}I using lactoperoxidase or using stoichiometric concentrations of hormone, chloramine T, and [^{125}I]sodium as has been described by Lesniak *et al.* for human growth hormone (*Nature New Biology* 241, 20, 1973). The ^{125}I-labeled hormone was purified by Sephadex G-100 chromatography to remove free ^{125}I, hormone aggregates, and iodinated subunits of the hormone. The [^{125}I]TSH had a specific radioactivity of 100 Ci/mM. Using the McKenzie bioassay, the thyrotropin stimulating activity of the TSH was 25 $\pm$ 4 U/mg and 21 $\pm$ 5 U/mg before and after iodination; the exophthalmogenic activity was 23 $\pm$ 6 U/mg and 22 $\pm$ 4 U/mg, respectively, using a fish bioassay. The ^{125}I-labeled hormone activated adenylate cyclase activity in thyroid membranes over the same dose response range as unlabeled hormone.

The [^{125}I]TSH binding to the thyrotropin receptors of purified bovine thyroid membranes was compared to the binding of biologically active [^{3}H]TSH. The binding of both labeled hormone preparations was similarly dependent on pH, time, temperature, and salt concentration and was proportional to the membrane protein concentration. Binding was not inhibited by insulin, glucagon, ACTH, and prolactin. Binding was negatively cooperative with an approximate affinity constant of 1 nM.

Similar studies with retro-orbital membranes show effectively identical results and show that the abnormal gamma globulin eliminates the negative cooperativity.

Binding to TSH receptors solubilized by treatment with triton or with lithium diiodosalicylate has also been studied. [^{125}I]- and [^{3}H]TSH binding were also cooperative but the affinity constant was lower. Binding specificity was altered with respect to glycoprotein hormones with analogous α subunits, *i.e.*, HCG and bovine LH. Similarly there were changes in salt and time dependence. It was concluded from these studies that both [^{125}I]TSH and [^{3}H]-TSH can be used to characterize TSH receptors and that solubilized receptors have altered properties. The receptor has been partially purified and is heterogeneous on sucrose density centrifugation. There are receptor components with molecular weights of 280,000, 150,000, 70,000 to 80,000, and 10,000 to 30,000. The latter species is > 30% carbohydrate, > 10% sialic acid, and is not destroyed by trypsin. A receptor component with similar properties has been isolated from tryptic extracts of thyroid cells in culture or from bovine thyroid membrane extracts.

The binding of TSH, LH, and their subunits to the TSH receptor of beef thyroid membranes has been compared to stimulation by these agents of adenylate

cyclase in the same membranes, glucose oxidation in dog thyroid slices, and the secretory process in mouse thyroids *in vitro* (colloid droplet formation) and *in vivo* (hormone release). The β subunits of TSH and LH can bind to the TSH receptor and can activate thyroid function *in vitro*. In contrast, the α subunit of TSH binds negligibly to the TSH receptor and has very low potency for stimulation of thyroid function (except for colloid droplet formation). Neither binding nor the biological activity of the β subunits can be accounted for by TSH contamination whereas this cannot be ruled out for α TSH. LH binds to the TSH receptor even better than the β subunit of TSH but the increased binding does not result in further activation of thyroid function. Neither α nor β TSH can alone account for more than 5-10% of the responses to intact TSH. It is proposed that the β subunit stimulates biological activity whereas the α subunit imposes conformational changes on the β subunit which promote binding *and* biological activity commensurably in the native TSH molecule but promote only binding in the native LH molecule.

II. Studies Concerning the Biosynthesis of Collagen. Dermatosparaxis is an inherited connective tissue disorder of cattle which is characterized by extreme fragility of the skin and an accumulation in the dermis of procollagen, the triple helical precursor of collagen which is composed of pro α1 and pro α2 chains. We have shown that pro α1 and pro α2 chains are ribosomal products which contain an additional peptide of 100 to 200 residues at the amino termini of their respective α chains. In addition, we have described an enzyme activity in normal bovine tissues which excised the amino terminal extension of the precursor chains to yield electrophoretically normal chains. This activity is extracellular and is absent or markedly reduced in dermatosparaxis tissues and in fibroblast cultures of several patients with variants of the Ehlers-Danlos syndrome, *i.e.*, the accumulation of procollagen and the resultant tissue changes were apparently caused by a defect in a specific enzyme, "procollagen peptidase."

We have described the purification of calf tendon procollagen peptidase, have characterized several of its properties, and have shown that the enzyme is an endopeptidase which excises the amino terminal peptide of procollagen in block rather than in multiple fragments. We have developed immunological assays of procollagen and procollagen peptidase which have allowed us to study the synthesis of these molecules *in vitro*.

Using the antibody to procollagen, we have shown that the cleavage product of procollagen peptidase, *i.e.*, the amino terminal peptide of procollagen, is present in the amniotic fluid and fetal sera of animals. The peptide has been purified from amniotic fluid and is being characterized for sequence analysis. In addition, we have purified the precursor form of fetal collagen (fetal procollagen) from amniotic fluid, have developed antibodies to it, and have initiated sequence studies in collaboration with the laboratory of Dr. George R. Martin (National Institute of Dental Research).

Joint biosynthetic and electron microscopic studies in fibroblasts have confirmed that procollagen is a ribosomal product which is packaged in intracellular vesicles and excreted into the media. Procollagen peptidase, although antigenically present in the intracellular vesicles and in early phases of the

excretory process, is enzymatically inactive either until procollagen concentrations in the media achieve a critical level or until the late log phase of cell growth is approached.

III. Studies Concerning Membrane Structure and Function. The membrane-bound D-lactate dehydrogenase of *E. coli* ML 308-225 has been solubilized and purified to homogeneity. Recent experiments demonstrate that guanidine extracts from wild type vesicles containing D-lactate dehydrogenase activity are able to reconstitute D-lactate-dependent oxygen consumption and active transport in membrane vesicles from an *E. coli* mutant deficient in D-lactate dehydrogenase.

When membrane vesicles prepared from a D-lactate dehydrogenase mutant of *E. coli* ML 308-225 are treated with a homogeneous preparation of D-lactate dehydrogenase, the enzyme binds to the vesicles and they regain the capacity to catalyze D-lactate oxidation and D-lactate-dependent active transport. Although membrane-bound enzyme increases linearly with addition of increasing quantities of enzyme, reconstituted transport activity and D-lactate oxidation are saturable functions of the amount of enzyme bound. The maximal specific transport activity obtained in the reconstituted system is similar in magnitude to that of wild type vesicles. Titration studies with 2-(N-dansyl)-aminoethyl-ß-D-thiogalactoside demonstrate that there is at least a 7- to 8-fold excess of *lac* carrier protein relative to D-lactate dehydrogenase. Hydroxybutynoate-inactivated enzyme does not bind to the vesicles, indicating that the coenzyme moiety is critically involved in binding. Conformational changes are also apparently involved since 0.6 M guanidine HCl is required for optimal binding and reconstitution. The relative unreactivity of reconstituted vesicles towards vinylglycolic acid suggests that D-lactate dehydrogenase is bound to the outer surface of the reconstituted vesicles.

An antibody to the enzyme has been developed. It precipitates enzyme and inhibits its activity. Native vesicles do not react with the antibody as measured by enzyme inhibition or altered transport; reconstituted vesicles react with the antibody as demonstrated by complete inhibition of enzyme activity and loss of transport. These studies also suggest a different structural location of the enzyme in native and reconstituted vesicles; direct experiments evaluating this point are in progress.

The membrane-penetrating MN-glycoprotein of the human erythrocyte has been further examined. Circular dichroism data analyzed by curve fitting techniques show that the intramembranous region of this glycoprotein forms an unusually stable helix when incorporated into egg lecithin liposomes. A computer search for hydrophobic regions in protein sequences suggests that the intramembranous domain of the MN-glycoprotein is exceptionally hydrophobic. These data support our previous contention that in the absence of strong perturbations, this intramembranous peptide segment should be helical in the membrane *in situ*. The length of this helix (35 Å) is of the order of thickness of the hydrophobic core of the membrane.

When isolated by trypsin digestion, the intramembranous segment contains bound cholesterol and phospholipid which can be removed with organic solvents.

In addition, this segment contains tightly bound phosphorous which is not associated with phospholipid.

IV. Structure Function Studies of Oxido Reductases. Oxido reductases which convert glyoxylate, hydroypyruvate, tartrate, and malate to glycolate, D(-)-glycerate, oxaloglycolate, and oxaloacetate, respectively, have been purified to homogeneity and characterized. Experiments have been directed towards understanding their mechanism of action, the mechanism by which they achieve specificity, and the mechanism by which their subunits associate to yield active enzyme. Present experiments involve sequence procedures of the active site of the induced and constitutive hydroxypyruvate reductases.

V. Centrifugation Studies. As a cooperating unit, ultracentrifugation studies have been performed this year for the following laboratories of this Institute: the Laboratory of Biochemistry and Metabolism (Drs. G. G. Ashwell, E. Cabib, and W. B. Jakoby), the Laboratory of Chemistry (Drs. C. R. Creveling and P. Torrence), the Laboratory of Biophysical Chemistry (Dr. D. R. Kominz), the Laboratory of Chemical Biology (Dr. A. N. Schechter), and the Clinical Endocrinology Branch (Dr. K. Ingham). Collaborative experiments with Drs. E. W. Miles and O. Adachi of this laboratory have shown that native $\alpha_2\beta_2$ and synthetic $\alpha_2\beta_2$ tryptophan synthetase have the same physical properties.

VI. Dishwasher Repair Unit. As a cooperating unit, emergency repair work has been performed on the Building 4 dishwasher.

Significance to Biochemical Research and the Progress of the Institute:

The research is aimed at evaluating enzymatic mechanisms by which cellular activities are regulated by pharmacodynamic agents. The possibility that unregulated biosynthetic or degradative processes may convert TSH into EPF active material has been established and a mechanism for human exophthalmos has been proposed on the basis of these data. A diagnostic method has been developed which predicts Graves' disease patients who will get exophthalmos during therapy and a therapeutic program to treat these patients is being preliminarily tested. Our studies of procollagen and procollagen peptidase in regard to collagen biosynthesis have become important in understanding fetal development and aging, as well as the pathogenesis of several connective tissue diseases. Diagnostic applications of our immunoassay procedures have been initiated in regard to the detection of nonafflicted carriers of several connective tissue diseases. Our enzyme studies of bacterial transport in *E. coli* have already led to the test of two enzyme inhibitors as potential antibiotics.

Proposed Course:

Studies of the TSH-exophthalmic factor relationship will be continued in an attempt to examine the mechanism by which EPF activity is generated *in vitro* and *in vivo*. The proposed diagnostic mechanism for human exophthalmos will be further tested as will the therapeutic program. The mechanism of procollagen peptidase formation and control will be related to normal and disease states. The biosynthesis of procollagen and fetal procollagen will be studied. The enzymatic mechanism of active transport in bacteria will be further

defined. Structure-function analyses of the reductase model system are being pursued. The localization of the active and regulatory sites on each enzyme will be sought and the comparative structures of these units will be compared. Comparison of membrane-bound and free enzymes will be made.

Honors and Awards: None

Publications:

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Short, S. A., Kaback, H. R., and Kohn, L. D.: Mechanisms of active transport in isolated bacterial membrane vesicles. XX. D-Lactate dehydrogenase binding in *Escherichia coli dld*$^-$ membrane vesicles reconstituted for active transport. *Proc. Nat. Acad. Sci. U.S.A.*, April 1974.

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Winand, R. J., and Kohn, L. D.: Retrobulbar modifications in experimental exophthalmos: the effect of thyrotropin and an exophthalmos-producing substance derived from thyrotropin on the $^{35}SO_4$ incorporation and glycosaminoglycan content of Harderian glands. *Endocrinology* 93: 670-680, 1973.

Winand, R. J., and Kohn, L. D.: The pathogenesis of human and experimental exophthalmos. *Excerpta Med. Int. Congr. Ser.*, in press.

Wolff, J., Winand, R. J., and Kohn, L. D.: The contribution of TSH subunits to the binding and biological activity of thyrotropin. *Proc. Nat. Acad. Sci. U.S.A.*, June 1974.

Serial No. NIAMDD-LBP-12
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through January 19, 1974

Project Title: Role of Subunit Interactions in Enzyme Chemistry

Previous Serial Number: Same

Principal Investigator: Dr. Claude B. Klee

Other Investigators: None

Cooperating Units: Drs. Louis A. Cohen and Kenneth L. Kirk, Laboratory of Chemistry, and Dr. Peter McPhie, Laboratory of Biochemistry and Metabolism, National Institute of Arthritis, Metabolism, and Digestive Diseases

Man Years:

Total:	1.2
Professional:	0.5
Others:	0.7

Project Description:

Objectives:

To identify the chemical structure of the active site residue of histidine ammonia-lyase and the participation of the enzyme subunits in the formation of the catalytic center.

Methods Employed:

The project involves the study of properties of proteins, purified by the usual techniques of protein isolation, including many types of chromatography. The enzymes are being studied by examining their optical, hydrodynamic, and kinetic properties as well as by suitable chemical measurements and modifications.

Major Findings:

I. Histidine Ammonia-Lyase. Histidine ammonia-lyase catalyzes an α,β elimination of NH_3 from histidine to produce urocanic acid. The active center of the enzyme contains an electrophilic center, as yet not characterized, which interacts with the α amino group of the substrate. Our present studies

are directed toward the elucidation of the structure of the active center and of the mechanism of the elimination reaction.

Active Site Structural Studies. Active site labeling of histidase with Na[^{3}H]BH_4 results in the incorporation of 3-4 moles of ^{3}H/mole of enzyme tetramer, and 50% of the bound ^{3}H is recovered as [^{3}H]alanine after acid hydrolysis. Exposure of the labeled enzyme to denaturing conditions prior to acid hydrolysis resulted in a loss of [^{3}H]alanine (< 0.4 moles/mole of enzyme), indicating that the dehydroalanine postulated at the active center is not simply linked to the protein by a peptide linkage.

We previously described a stereospecific inactivation of the enzyme by L-cysteine which is accompanied by the binding of 3.2-3.5 moles of cysteine per mole of enzyme tetramer and by the subsequent appearance of a new absorption peak at 340 nm. The protection observed in the presence of L-histidine, urocanate, but not D-histidine, indicates that the modification is active site specific. Preliminary evidence indicates that cysteine acts as a substrate analog interacting with the enzyme through its amino group. Binding of cysteine is followed by a chemical change responsible for the new absorption peak. The structure responsible for this absorption does not include the added cysteine since the cysteine may be dissociated from the enzyme under denaturing conditions or extremes of pH without loss in absorption at 340 nm. A 10-15 residues peptide containing the new chromophore but not the labeled cysteine has been purified and is presently being analyzed. A Schiff base between the amino group of dehydroalanine and an unknown carbonyl function has been postulated by Givot, Smith, and Abeles. It is possible that such a carbonyl group may be involved in the reactions of the enzyme with cysteine, but further studies are required before any such inferences may be confidently made.

Mechanism of Action. Our investigation of the β elimination of NH_3 from L-histidine and 4-fluoro-L-histidine by histidine ammonia-lyase has been extended to include 4-NO_2-L-histidine. Under comparable conditions the V_m for L-histidine, 4-fluoro-L-histidine, and 4-NO_2-L-histidine were 20, 0.5, and 1.7 μmole/min/mg, respectively, and the K_m were not significantly different. The steady state kinetics previously reported indicated that the rate determining step precedes the dissociation of ammonia from the enzyme. An isotope effect of 1.5 to 1.8 observed with DL-[β-$^2H_2$]histidine and 4-fluoro-DL-[β-$^2H_2$]histidine suggests that the C-H bond breakage is at least partially rate determining for these two substrates. No isotope effect is observed in the deamination of 4-NO_2-DL-[β-$^2H_2$]histidine in which the β hydrogen is labilized by the substituent in the 4 position of the imidazole ring. The rate limiting of the deamination of 4-NO_2-L-histidine is therefore different from that of histidine or 4-fluoro-histidine. This conclusion is confirmed by the absence of inhibition by EDTA observed with 4-NO_2-histidine in contrast to the metal activation of the deamination of L-histidine which has been attributed to the labilization of the β hydrogen.

II. Urocanase. The degradation of the urocanic derivatives obtained by enzymatic deamination of the histidine analogs described above by urocanase, the next enzyme in the degradative pathway of histidine has been investigated.

Fluorine substitution in the 4 position of the imidazole results in complete loss of activity. On the contrary, fluorine substitution in the 2 position results in a greatly increased affinity ($K_i = 10^{-7}$, $K_m = 10^{-6}$ M) but a 100-fold decrease in V_m. The existence of a tightly bound enzyme substrate intermediate can be a very useful tool to study the mechanism of the reaction catalyzed by this enzyme.

Significance to Biomedical Research and the Program of the Institut

Histidine ammonia-lyase and phenylalanine ammonia-lyase are the only enzymes so far known to have unsaturated amino acid residues as part of their active sites. Doubtless, other such enzymes will ultimately be described and an understanding of the mechanism of action of the one protein will be of use in understanding the mode of action of the whole class. The mechanisms by which such unsaturated amino acids arise, both in proteins and in some peptide antibiotics, are open questions but may well have implications for the structure of some cross-linked proteins. Dehydroamino acids formed as a result of the elimination of various groups on the β carbon of amino acid residues may be important in structural as well as functional roles.

Proposed Course:

Future work on histidine ammonia-lyase will be directed toward the characterization of the chemical structure of the electrophilic center and of its function in the mechanism of the catalysis of the α,β elimination.

These studies will be pursued in the Laboratory of Biochemistry, Division of Cancer Biology and Diagnosis, National Cancer Institute, National Institutes of Health.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LBP-13
1. Biochemical Pharmacology
2. Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanisms of Inheritance in *Saccharomyces cerevisiae*

Previous Serial Number: None

Principal Investigator: Dr. Reed B. Wickner

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1.3
Professional:	1.0
Other:	0.3

Project Description:

Objectives:

The long-term objectives are: (I) to understand the mechanisms of control of inheritance and expression of the killer plasmid and (II) to understand the mechanisms whereby chromosomal inheritance is controlled.

Methods Employed:

I. A killer strain was mutagenized and strains showing abnormal killing characteristics were isolated and characterized by standard genetic techniques. Methods are now being devised to purify the plasmid genome in high yield in order to study alterations in the genome resulting from various mutations obtained.

II. Standard biochemical and genetic techniques have been used.

Major Findings:

I. *The "Killer Character" of Saccharomyces cerevisiae* -- The "killer" character" of the eukaryote *Saccharomyces cerevisiae* is an extrachromosomal genetic element which is associated with two discrete double-stranded RNA species and virus-like particles.

I have isolated mutants in two chromosomal genes whose functions are essential for maintenance of the extrachromosomal killer genome (mak).

One of these (mak-1) is located within 2 centimorgans of the centromere of chromosome XV. Two other chromosomal genes are needed for expression of the killing function (kex). One of these (kex-2) is located on chromosome XIV by disome analysis, but is not meiotically linked to any of the known markers on chromosome XIV. Another gene (rex) is needed to express resistance to the extracellular killer substance, and since mutants in this gene still express killing, the cells can commit suicide. Both kex and rex mutants still harbor the complete cytoplasmic killer genome.

Mutations in the extrachromosomal killer genome have also been obtained. One type has lost killing ability but retains resistance to killing and is recessive to wild type. A second type of mutant has lost both killing ability and resistance and is recessive to wild type, but introduction of the wild type killer plasmid results in selective loss during mitosis of the wild type genome and retention of the mutant plasmid. This phenomenon is called "suppressiveness." A third type of mutant may lack killing ability or resistance or both and is recessive to the wild type. It has the interesting property that mating with any strain, even one apparently lacking the plasmid, restores the wild type phenotype. This suggests that these mutants carry the plasmid in some sort of "latent" state.

I have also found that growth of killer strains at elevated temperatures results in the loss of part or all of the plasmid genome.

II. *DNA Replication in Saccharomyces cerevisiae* -- I have selected mutants of yeast capable of dTMP uptake and incorporation specifically into DNA. Since DNA cannot be specifically labeled in yeast by either thymine or thymidine, these strains will be of great value in the study of DNA metabolism of yeast. These strains also incorporate dBrUMP into DNA. These mutants fall into four complementation groups. One group, tup-1, located on chromosome III, shows abnormal mating behavior when in conjunction with the α mating type. In addition homozygous a/α tup-1/tup-1 strains fail to sporulate.

Significance to Biomedical Research and the Program of the Institute:

I. The killer plasmid appears to be composed of two species of double-stranded RNA and is associated with virus-like particles. While double-stranded RNA viruses are ubiquitous, infecting bacteria, fungi, plants, insects, and mammals (including at least two pathogens infecting man), this is the first example of a plasmid with a double-stranded RNA genome. The distinction may, however, be purely formal. This system provides a simple means of investigating host-virus genetic relationships as well as the mechanisms of segregation, recombination, and expression of such plasmids.

II. The mutants I have isolated which incorporate dTMP into DNA have already been used by several other groups in their studies of DNA replication and structure in yeast.

Proposed Course:

(1) I found that growth at elevated temperatures efficiently "cures" cells of their killer plasmid. Isolation of mutants which cannot be cured

by this procedure is likely to reveal interesting features of the mechanisms by which the plasmid is maintained.

(2) The mutants already obtained are being further characterized with respect to their double-stranded RNA content.

(3) While some killer strains can give rise to "suppressive" mutants, others are unable to do so. The genetic basis for this strain difference is now under investigation.

(4) Studies of segregation in meiosis of the extra chromosome of trisomic strains are now underway.

(5) Attempts to demonstrate auxiliary pathways of mitosis are in progress.

Honors and Awards: None

Publications:

Wickner, R. B.: Chromosomal and nonchromosomal mutations affecting the "killer character" of *Saccharomyces cerevisiae*. *Genetics*, March 1974.

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ANNUAL REPORT SUMMARY

LABORATORY OF CHEMICAL PHYSICS

Before summarizing the major research findings during the last year, it is appropriate to point out that the International Union of Astronomy (IUA) recently named one of the craters on the Moon in honor of Dr. Frederick Brackett, for many years a Research Biophysicist at NIH and a consultant to our Laboratory since his retirement in 1961. Dr. Brackett is well known as a pioneer in establishing research in biophysics at NIH and later played a key role in introducing digital computers here. But he had earlier gained renown for his discovery of the Brackett Series in the spectrum of atomic hydrogen--a discovery of great significance in the verification of quantum theory and of considerable utility in astrophysics. It is, of course, this latter aspect that prompted the honor by the IUA.

Current research in the Laboratory is heavily dependent on the development of new spectroscopic methods and the application of those methods to the elucidation of molecular structure and molecular interactions. Such research generally proceeds along several lines: (1) analysis of the structure, electronic states or force fields of small molecules; (2) studies of the physical properties of biological macromolecules as related to their biological activity and function; and (3) refinement of techniques to permit more detailed and sensitive investigations of these phenomena.

In the study of small molecules, Kon (in cooperation with Szent-Gyorgyi of the Woods Hole Biological Laboratories) has continued work on charge-transfer transitions and now finds that a normally short-lived charge transfer complex formed in strong acid solution between p-phenylene diamine and glyoxal can be trapped permanently in the solid state by reacting the dry complex with HCl gas. In another study the electron paramagnetic resonance spectra were found to be extremely sensitive to the coordination of the solvent with an NO complex of Fe(II) mesotetraphenyl-porphine, which is a model for the NO derivatives of heme proteins. With this information it has become possible to interpret some of the epr spectra, which have been widely observed, of the NO derivatives of structurally perturbed hemoproteins.

Along similar lines, using a different technique, Eaton and Hanson are studying the optical spectra of pure porphyrin and metalloporphyrin single crystals as models for understanding heme-heme interactions in multiheme proteins. The first study on the metal-free porphyrin has uncovered a low intensity transition out-of-plane of the porphyrin ring. This may be the first observation of an optical transition involving sigma electrons in a free base porphyrin. Charney and Rosenfield have begun an examination of the natural and magnetically induced optical activity of conjugated diketones. This is an extension of earlier work by Charney (with Tsai, NHLI) on the spectroscopic states of these molecules and has been prompted by a whole new series of studies initiated recently in various other laboratories since the publication of the original work.

McDiarmid has continued studies of the sulfur analogues of ether, and for dimethyl sulfide she has assigned the approximately 25 bands at higher energies than 2500 Angstroms to the three Rydberg series, that is, to electronic promotions to atom-like electronic orbitals. In addition, in dimethyl ether itself a previously uninterpreted transition at 1700 Å has been assigned as a promotion from an orbital lower in energy than the highest filled, just as previously found in the sulfur analogue.

Levin, Pearce and Spiker observed Raman spectra of dimethyl sulfide and its fully deuterated analog and used these data in establishing a detailed vibrational assignment and inferring an intramolecular force field. This force field emphasizes the contribution to the potential function of a small but significant methyl-methyl coupling term between the anti H-C-S angular vibrational coordinates. Similar work by Levin and Harris on polycrystalline quadricyclene, a highly strained ring system, has established probable "characteristic frequencies" for skeletal modes in a variety of similar strained ring molecules.

Nuclear magnetic resonance (NMR) is one of our most powerful spectroscopic tools for the elucidation of molecular structure, especially since the introduction of pulse and Fourier transform methods to permit measurement of nuclear relaxation times and to facilitate the acquisition of NMR data from insensitive nuclei such as ^{13}C. During the last year Wasylishen and Cohen found that the spin-lattice relaxation time of protons in imidazole is markedly influenced by the pH-dependent complexation of minute amounts of paramagnetic ions. A complete understanding of this process is essential to avoid misinterpretations of similar experiments in proteins, where paramagnetic impurities may be present. Meanwhile, Tucker and Becker investigated the effect of hydrogen bonding on the ^{13}C relaxation time of the carbinol carbon in tert-butanol. They found an increase of a factor of six as the hydrogen bonded aggregates are broken on dilution in an inert solvent, and they thus proposed that such relaxation time measurements can serve as a sensitive indicator of the degree of hydrogen bonding in a system undergoing complex multiple equilibria.

Wasylishen conducted concurrent experimental and theoretical (molecular orbital) studies of some of the factors affecting both nuclear spin-spin coupling between ^{13}C and ^{15}N in a variety of molecules and chemical shifts of ^{13}C in a series of isothiazoles. Ziffer used ^{1}H and ^{13}C NMR in the elucidation of the stereochemical structure of 3-methylcyclohexane-1,2-diols. Rice, Weiss and Wasylishen made a careful study by NMR of the conformational mobility in hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one, while Weiss and Rice used a variety of instrumental methods to unravel the structure of a complex (C_{17}) molecule formed (surprisingly) in one step under mild conditions from the reaction between dimethyl β-ketoglutarate and glyoxal in aqueous solution. Ziffer used a combination of spectroscopic methods to determine the absolute stereochemistry of a particular microbial metabolite and of two trans p-mentha-2,3-diols. He also found that the relative rates of the singlet and triplet paths for photochemical decomposition of a naphthalene-2-one are dramatically altered by introduction of a methyl group on the α-carbon.

In the study of macromolecules by spectroscopic and other physical methods, Eaton (with Padlan, LMB and Yonetani, University of Pennsylvania) in combined

x-ray and optical crystallography studies have examined the major differences between cobalt substituted myoglobin and acid metmyoglobin and found differences in metal coordination number and heme orientation which may be related to the role of the metal in the stereochemical trigger that is responsible for cooperativity in oxygen binding by these proteins. Kon, continuing his study with Nebert (NICHD) of the cytochrome P450 system in liver microsomes by electron paramagnetic resonance, has found that a genetically mediated process is involved in the increase of the high spin moiety of the cytochrome compared to the low spin moiety on in vivo treatment of rat and mouse with 3-methyl-colanthrene. Charney (with Milstien, FDA) using electric dichroism relaxation methods has found that the flexibility of double stranded Poly rA is considerably greater than that of DNA. This result may have some bearing on the role of Poly rA stretches in many RNA's.

Eaton and Hofrichter (with Ross, LMB) has been studying the aggregation (gelation) of deoxyhemoglobin S. It is this process which distorts red cells upon deoxygenation and results in the crises of sickle-cell disease. The heat capacity, viscosity, optical birefringence and distribution of hemoglobin between the gel and liquid phases are under study. The gelation process which appears to take place spontaneously under appropriate conditions exhibits an enthalpy change of less than 2700 cal/mole hemoglobin suggestive of a process involving weak intermolecular interactions and small conformational changes.

Charney (with Yamaoka, University of Hiroshima and Milstien, FDA) has continued the studies of the electric dichroism of double stranded DNA and polyriboseadenylic acid, in both cases with molecules short enough so that their rod-like character is established both from hydrodynamic and now from electric dichroism studies. Ambiguities arising from molecular flexibility therefore do not arise. In the case of DNA, the study confirms the earlier result that a significant component of the nucleoside base transitions near 260 nm is polarized along the helix axis. For Poly rA, it has been shown that the flexible molecule is extended by the electric field to the same rod-like conformation as the shorter molecule.

Levin and Spiker have undertaken a laser Raman study of conformational changes in lecithin multilayer and vesicle bilayer model membrane systems. They made a detailed assignment of the Raman bands in order to establish which frequencies are characteristic of the choline, phosphate and hydrocarbon moieties. With this background they used precise frequency shift measurements to locate the gel-liquid crystalline transition temperatures and to probe the changes occurring in chain fluidity when other components, such as cholesterol, were added to the system.

Shean and Sollner are approaching the problem of model membranes in a different manner--through the study of liquid ion exchanger membranes. Their recent results indicate that the Na^+, K^+ bi-ionic potentials across membranes consisting of dipicryl amine (DPA) in o-nitrotoluene are essentially independent of the amount of DPA in the membrane. They found also that the particular solvent employed in the membrane had a pronounced effect on ion preferences across the membrane.

Development of new spectroscopic methods and refinement of existing methods to improve versatility, sensitivity or ease of use forms an important part of our research program. During the last year Gupta and Becker (with Ferretti, DCRT) made a careful study of the theoretical basis for and the extent of applicability of a recently suggested technique--rapid scan correlation NMR. This method complements pulse methods and seems to be particularly advantageous in the study of NMR spectra of samples (such as proteins) in aqueous solution at low concentration.

Many instrumental techniques now rely on direct access to a sophisticated digital computer. During the last year the Honeywell H516 data-acquisition computer system in Building 2 has been further expanded by Jennings, together with several individuals from DCRT. A Cary 118 UV spectrophotometer used in thermal melting studies on nucleic acids by Miles and associates (LMB) and a microspectrophotometer for protein crystal studies by Eaton and Hofrichter have been added to the computer system. A new cathode-ray display terminal and many new general-purpose user programs have been added to assist in processing files of data collected from laboratory instruments. Files can now be interchanged by telephone line with the 360-370 computer systems in Building 12. During the past year several publications by members of the Laboratories of Chemical Physics and of Molecular Biology have used data gathered, processed, and/or prepared in graphical form on the H516 system.

Studies of the mechanism of visual excitation are being pursued by several scientists in the Laboratory. Structural studies of the rhodopsin chromophore by Adams have continued. Since current evidence indicates that the retinaldehyde moiety of visual pigments is bound by a Schiff-base linkage to the ε-amino group of a lysine in a peptide chain, attempts have been made to find model peptide sequences containing lysine that have the same spectral-shift-inducing properties on the retinaldehyde spectrum as active opsin. Quantum theoretical calculations by Sharpless, Jennings and Adams indicate that a charge transfer model for the retinaldehyde spectral shift is consistent with what little is known about the chemical structure of the rhodopsin chromophore.

The question of how the photochemical action of light on rhodopsin is translated into sensory excitation has been pursued through studies of the ionic permeabilities of cell membranes of living retinal rod cells and of isolated rod outer segments. Yoshikami and Hagins have shown that the antibiotic X 537A, which is a specific carrier of calcium ions through cell membranes, greatly sensitizes living rods to Ca^{++} applied in bathing solutions. The action of applied Ca^{++} on the sensory dark current of rods is quite similar to that of light and further supports the hypothesis that Ca^{++} is an intracellular sensory transmitter substance mediating visual excitation. Rice and Sharpless have done preliminary work on the synthesis of intracellular calcium-sensitive spin labels to be used in studying the role of divalent ions in visual excitation.

Cobbs, Robinson, Yoshikami and Hagins have begun to investigate the permeabilities of the constituent membranes of isolated retinal rod outer segments. A fluorescent probe, N, N' di DANSYL cystine (DDC) was found to differentiate between outer segments that possess an intact plasma membrane

and those that consist only of bare membranous disks. Using a combination of isotopic tracer techniques, chemical analyses, and osmotic perturbation experiments together with the new DDC assay it has been found that the plasma membranes of isolated frog rods are almost impermeable to chloride ions and impermeable to ATP. Nevertheless, there is more than 1 mM ATP in isolated rods, and it is half consumed in 30 min.

Turning finally to the largest systems studied in the Laboratory, intact animals, Altland has found that in shaven rabbits exposed to cold (-5°C) about one-half of the animals became severely hypothermic. In these cases glycolytic and oxidative enzymes in the serum reached peak concentrations within the first day after exposure at a time when fatty changes and necrosis were found in the heart and other organs. The study showed that it is important to make serum enzyme determinations immediately after cold exposure and at daily intervals thereafter in order to aid in the diagnosis of tissue changes. The serum enzyme changes usually persist for only 24 hours.

On April 22, 1974, the "altitude chamber," which had been in continuous use by the Physiology Section since 1941, was removed from NIH in order to raze Temporary Building T-7 in which it was housed. During World War II the "altitude chamber" was first used to test the effectiveness of experimental aviation breathing equipment for the U.S. Navy at simulated altitudes up to 42,000 ft. at temperatures down to -40°F. For several years after the war man tests were conducted to determine the effects of simulated altitudes upon breath velocity patterns. After 1948 animal experimentation concerned with a variety of physiological problems were conducted such as the effect of high altitude upon length of life, reproductive performance, resistance to X-irradiation, kidney functions, myoglobin, and cardiovascular abnormalities. During this period a method was developed by use of the "altitude chamber" for the production of experimental valvular heart disease. Some of the work which had been done in the large altitude chamber has been transferred to and will continue in the small altitude chamber in Building 2.

Serial No. NIAMDD-LCP-1
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The physical and chemical bases of photoreception

Previous Serial Number: NIAMDD-LCP-2

Principal Investigator: W. A. Hagins

Other Investigators: W. H. Cobbs, III
W. E. Robinson

Cooperating Units: Hartmann Rüppel (Max Volmer Institute, Berlin, Germany)
Frances M. Hagins (Guest Worker, LCP-NIAMDD)
Shuko Yoshikami (Guest Worker, LCP-NIAMDD and NEI-LVR)

Man Years

Total:	4
Professional:	4
Other:	0

Project Description:

Objectives:

To outline the successive events by which light quanta absorbed in the receptors of animals lead to the transmission of information to the brain.

Methods Employed:

1. Measurements of currents, voltages, and impedances of the retina by conventional electronic methods.
2. Measurement of photochemical changes in rhodopsin in living cells by kinetic microspectrophotometry.
3. Analysis by digital computation of electrical signals produced by photoreceptors.
4. Investigation of the chemical structure of rhodopsin by spectrophotometry and amino acid analysis.
5. Electron microscopy of the cellular structure of photoreceptors.
6. Analysis of the ionic composition of the retina by electron probe microanalysis.
7. Study of the enzyme activity and ionic fluxes in isolated photoreceptive organelles.

Major Findings:

1. The dark current of living retinal rod receptors, which has been previously shown to be specifically and reversibly suppressed by light during sensory transduction, is also suppressed by low concentration of Ca^{++} ions applied to the exterior of the cells. When the plasma membranes of these cells are exposed to traces of the antibiotic X 537A, which makes membranes permeable to Ca^{++}, externally applied Ca^{++} becomes some 2000 times more effective in suppressing the dark current. This is important evidence supporting an hypothesis previously proposed by our group that internally released Ca^{++} ions are sensory transmitters essential in visual transduction. (Yoshikami and W. A. Hagins)

2. A study of the ionic permeability of the light-sensitive phospholipid membranes of retinal rod outer segments (o.s.) has begun. o.s. can be broken from the surface of isolated frog retinas to form free-floating guasi-crystalline bodies. By using the fluorescent probe, N, N'di DANSYL cystine (DDC) as a selective stain, we have shown that the isolated o.s. are of two types: those enclosed in a plasma membrane (Type N) and those that consist of naked stacks of flattened rhodopsin-bearing phospholipid sacs, (Type P). Type N o.s. shrink like osmometers in hypertonic salt solutions but do not stain with DDC, type P ones do not shrink but bind DDC strongly. Using the DDC staining technique to assay the population of o.s. with intact plasma membranes, the permeability of rod plasma membranes to Cl^- ions has been measured, and found to be very low. This finding has invalidated a claim published by another laboratory outside NIH that changes in NaCl permeability of of isolated o.s. can be observed by osmotic methods. (Cobbs, Yoshikami, Robinson, and W. A. Hagins)

3. A method has been developed for measuring ATP consumption by freshly isolated frog o.s. The cytoplasmic ATP concentration is initially much like that of skeletal muscle but it declines with a half-time of $\sim$30 min. Light seems to increase ATP utilization slightly. (Yoshikami, Robinson, and W. A. Hagins)

Significance to Biomedical Research and the Program of the Institute:

Progress in the understanding of molecular events underlying excitation of photoreceptors is obviously important in research in vision. In addition, it may provide a point of attack on the nature of permeability changes which occur in the surface membranes of electrically active cells, such as nerve and muscle, during transmission of nerve impulses and initiation of muscular contraction.

Proposed Course of Project:

1. Using a new group of experimental techniques developed during the past year we shall investigate the permeabilities of isolated rod outer

segment membranes to the small anions and cations, particularly Ca^{++} ions, that play a part in sensory excitation in nerve and muscle cells.

2. Following reports in the literature that rhodopsin is phosphorylated when exposed to light, we are studying the question of whether phosphorylation of membrane-bound proteins in rods is an essential step in visual excitation.

Publications:

Hagins, W. A., and Yoshikami, S.: A role for calcium in visual excitation. Exp. Eye Res. (in press).

REPORTED IN PRESS - 1973

Yoshikami, S., and Hagins, W. A.: Control of the dark current in vertebrate rods and cones. In Langer, H. (ed.): Biochemistry and Physiology of Visual Pigments. Berlin, Germany, Springer-Verlag, 1973, pp. 245-255.

Rüppel, H., and Hagins, W. A.: Spatial origin of the fast photovoltage in retinal rods. In Langer, H. (ed.): Biochemistry and Physiology of Visual Pigments. Berlin, Germany, Springer-Verlag, 1973, pp. 257-261.

Hagins, F. M.: Purification and partial characterization of the protein component of squid rhodopsin. J. Biol. Chem. 248: 3298-3304, 1973.

Serial No. NIAMDD-LCP-2
1. Chemical Physics
2. Membrane Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Digital computer facilities for LCP and LMB

Previous Serial Number: NIAMDD-LCP-3

Principal Investigator: William H. Jennings, Jr.

Other Investigators: None

Cooperating Units: Computer Systems Laboratory, DCRT

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

Development of time-sharing computer facilities for on-line data acquisition and processing from various computers and scientific instruments in Building 2.

Methods Employed:

Computer software development and testing; interfacing laboratory apparatus with the time-sharing computer.

Major Findings:

The beginning of fiscal 1974 was marked by severe problems in obtaining satisfactory service for the Honeywell 516 equipment. Several meetings with Honeywell Field Engineering personnel established lines of communication and changes in service procedures which now provide rather good service.

Two new instruments became fully operational on-line this year: the Cary 118, UV spectrophotometer and the Micro-spectrophotometer of Drs. Eaton and Hofrichter. In addition, a digital temperature meter was added to the Beckman IR-7 interface and a new Remote Operators Console (ROC), with dual instrument capability replaced the existing Cary 14 ROC. This dual capability will be used in the future by Dr. McDiarmid to transfer data from a

local memory in a stand-alone system connected to a vacuum UV spectrophotometer.

The only change in system equipment this year was the addition of a Tektronix 4012 Graphics terminal. This keyboard entry and CRT display device operates at a very high speed and in many cases provides a desirable alternative to the line printer for output. The basic graphics subroutines and an extensive terminal test program have been written and tested for this device. A special-purpose Polaroid camera was purchased to make photos of the CRT display.

Almost all utility programs i.e., programs which transfer data from one device to another or perform file maintenance functions, were revised during the year. The purpose of this upgrading was: (1) To remove the use of Sense Switches from these programs, because this use interfers with reliable operation of the data acquisition programs. (2) To improve the dialogue between machine and man. This was facilitated by the high speed of the Tektronix 4012 which permits more verbose instructions and questions. (3) To provide a switch between normal output to the line printer and optional output to the Tektronix terminal. Where no permanent record is needed, the terminal output is faster. This option can be very important to continued operation of the system in the event of hardware failure of the printer or in the very possible event that paper may be temporarily unavailable. (4) To improve the format of the printed output from file manipulation programs. This greatly reduces the amount of paper used as well as that required to be stored for future reference.

Approximately two dozen new user programs of all types were added to the program library during the year. The availability of several programs that will manipulate tables larger than 1000 points has lead to a demand for further programming with this capacity and in the process of revision, a considerable upgrading of programming is achieved. For example: a linear smoothing program was rewritten with size limits removed and in a general form which permits addition of special smoothing algorithms in a straight-forward manner. Although specialized processing programs are necessary, most programs on this system are sufficiently general as to be useable by most investigators.

Significance to Biomedical Research and the Program of the Institute:

On-line digital data acquisition from laboratory instruments has become an essential element in the research projects of some investigators in LMB and LCP. The capacity to rapidly process, compare and display large data files, particularly in the case of short-lived preparations, allows these investigators to work with data bases not ordinarily practicable. In some cases the availability of this computer system permits important experiments that would normally be considered so tedious as to be marginally useful.

Proposed Course of Project:

A major revision of the computer's operating system will be undertaken in the late summer when it may be possible to remove the system from service for several weeks. This revision will incorporate newer Honeywell programming and routines from the Dental Institute in order to increase efficiency and reliability of handling simultaneous users. The upgrading of existing programs will continue with particular emphasis on well documented programming using standard subroutines to permit simplified software maintenance. It is anticipated that much of the new programming for users will use the interactive graphics capability of the Tektronix 4012 terminal.

With few exceptions, the hardware components of the system are in good condition and appear completely adequate into the foreseeable future. An important exception is the Process Interface Controller (PIC), which interfaces the instruments to the computer. A thorough review of the repair, maintenance and upgrading aspects as well as possible replacement of this device is underway. One new instrument is being considered for interfacing to the system this next year. The Varian C-1024 signal averaging computer (formerly used in conjunction with an NMR device) may be supplanted by Dr. Hideo Kon's electron paramagnetic resonance instrument. As mentioned last year, new instruments can be added only if presently connected ones are removed.

Publications:

Sharpless, N. E., Adams, R. G., and Jennings, W. H.: Retinal Schiff bases. The effect of water on spectral absorption. Trans. N. Y. Acad. Sci. 35: 547-552, 1973.

Adams, R. G., Sharpless, N. E., and Jennings, W. H.: Some aspects of the Schiff bases of retinaldehyde. J. Expt. Eye Res. 18: 13-17, 1974.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Spectral, physical-chemical and photochemical properties of biologically active substances

Previous Serial Number: NIAMDD-LCP-4

Principal Investigator: Norman E. Sharpless

Other Investigators: None

Cooperating Units: Frederick S. Brackett, Consultant, LCP-NIAMDD
Ralph G. Adams (LCP-NIAMDD)
William H. Jennings (LCP-NIAMDD)
Ulrich Weiss (LCP-NIAMDD)

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To investigate such molecular factors as structure, quantum parameters or physical properties in relation to the biological or pharmacological activities of the molecules.

Methods Employed:

These investigations utilize various forms of spectrophotometry (visible, UV, IR, NMR, EPR and fluorescence) which distinguish energy levels, and ORD, CD, gas chromatography and dielectric properties which distinguish other molecular properties, as well as theoretical quantum chemical calculations. Extensive use of computers is required to reach these objectives.

Major Findings:

Photochemistry of the Vitamin D System (with Dr. Brackett)

Continuing analysis of the yields of products of the irradiation of various members of the series indicates that thermal reactions are of

utmost importance in the sequence, and have previously been ignored by most investigators.

Chemistry and Physics of the Visual Process (with Dr. Adams and Mr. Jennings)

Quantum calculations on models of the Schiff bases formed by retinaldehyde with either ethanolamine sulfuric or phosphoric acids indicate that the phosphoric acid derivative is the less stable. This may be a factor in the different reactivities of these two compounds toward water.

Work has continued on the synthesis of the tripeptides lysylalanylphenylalanine as a partial model for the protein opsin.

Many of the properties of retinaldehyde appear to be related to its great ability to form acetals. We have synthesized the highly hindered alcohol tri-t-butylcarbinol. This compound is unable to form an acetal with retinaldehyde, and the formation of a Schiff base seems slower in its presence. The exact cause is at present uncertain.

Therapeutic Interference Studies

Coupling a spin label with acetylacetone to give a labeled molecule capable of chelating calcium ion, utilizing the thallium derivative in N,N-dimethylformamide gave rise to the enol ether rather than the C-alkylated derivative desired. However, conducting the reaction in dioxane gives rise to the proper product, N-(3-(2,2,6,6-tetramethylpiperindooxyl)-1,1-diacetoxypropionamide).

An alternative compound, N-(3-(2,2,6,6-tetramethylpiperindooxyl)-1-acetoacetamide has been synthesized and also shows promise in this regard. This acetamide derivative apparently complexes copper, with a dimunition of the ESR signal.

Additional Structural Studies (with Dr. U. Weiss)

The confirmations of the structures of two isomeric triketones were obtained by the use of dipole moment measurements, permitting the interpretation of their NMR spectra.

Significance to Biomedical Research and the Program of the Institute:

A proper understanding of the behavior of biologically active substances at the molecular level is absolutely necessary for the understanding and interpreting of their action at the cellular and organ level in order to evaluate their contributions to both healthy and diseased states.

Proposed Course of Project:

The study of the interactions of retinaldehyde with the model peptides will be continued, both in regard to structure and optical properties.

The spin-labeling of biologically important molecules will be continued.

Publications:

Sharpless, N. E., Ferretti, J. A., and Bradley, R. B.: The nuclear magnetic resonance of heterocyclic compounds related to anthracene. Org. Magn. Reson. 6: 115-120, 1974.

Adams, R. G., Sharpless, N. E., and Jennings, W. H.: Some aspects of the Schiff bases of retinaldehyde. Expt. Eye Res. 18: 13-17, 1974.

REPORTED IN PRESS - 1973

Sharpless, N. E., Adams, R. G., and Jennings, W. H.: Retinal Schiff bases. The effect of water on spectral absorption. Trans. N. Y. Acad. Sci. 35: 547-552, 1973.

Serial No. NIAMDD-LCP-4
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title:

1. π-π complexes of retinal with substances occurring in mammalian retina in vivo.
2. Construction of a model system to approximate the complex described and to determine a possible relationship to rhodopsin.

Previous Serial Number: NIAMDD-LCP-5

Principal Investigator: Ralph G. Adams

Other Investigators: None

Cooperating Units: W. H. Jennings (NIAMDD-LCP)
N. E. Sharpless (NIAMDD-LCP)

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

1. To determine the composition of the rhodopsin chromophore. Although we still consider our previously described complex II to be a prime candidate, recent reports of the absence of ethanolamines phosphoglyceride (EPG) in rhodopsin make essential the refutation of the evidence or finding an alternative candidate. To that end we are investigating lysine-containing peptides since in forming the required Schiff base only lysine and ethanolamine are considered.
2. The end point of a synthetic rhodopsin is still a valid objective and the route to that end is logically via the peptides, since rhodopsin is a lipo-protein.

Major Findings:

Dipeptides and tripeptides made by the "batch" method were too often impure and of small yield requiring change to the Merrifield resin method

which has involved some learning of techniques but is now paying off in better products. As yet we have not had a sufficiently pure tripeptide of the lysyl-alanyl-phenylalanine structure to eliminate the possibility of contaminant interaction with retinal. The present crop, however, promises to be sufficient. We have no evidence against our hypothesis of a charge transfer complex in the rhodopsin chromophore to date.

Recently we have evidence that acetal formation is a step in the formation of complex II supported by the need for the presence of methanol. Currently we are determining whether this may be so by substituting a tri-tertiary-butyl-carbinol for methanol. This required synthesis of that molecule has just been completed. We have not yet conducted the critical experiment pending purification of the carbinol which contains a carbonyl starting material.

Our ongoing computer (Sharpless and Jennings) research for a true model of rhodopsin is producing no startling results but each test result has thus far substantiated our concept of rhodopsin as a π-π complex between retinal and either EPG (ethanolamine phosphoglyceride) or a lysine containing peptide which also has phenylalanine in it.

Significance to Biomedical Research and the Program of the Institute:

To elucidate the structure of rhodopsin, the visual pigment is an obviously important step in understanding vision.

Proposed Course of the Project:

1. Application of results of kinetic studies to formation of model compounds approximating rhodopsin.
2. Formation of additional peptides to complex with retinal.
3. Rotatory dispersion and circular dichroism studies of synthetic peptide-retinal complexes.
4. Repeat other experiments with enzymes on rhodopsin.

Publications:

Adams, R. G., Sharpless, N. E., and Jennings, W. H.: Some aspects of Schiff bases of retinaldehyde. Expt. Eye Res. 18: 13-17, 1974.

REPORTED IN PRESS - 1973

Sharpless, N. E., Adams, R. G., and Jennings, W. H.: Retinal Schiff bases. The effect of water on spectral absorption. Trans. N. Y. Acad. Sci. 35: 547-552, 1973.

Serial No. NIAMDD-LCP-5
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Molecular structure determinated by spectroscopic methods

Previous Serial Number: NIAMDD-LCP-6

Principal Investigator: Edwin D. Becker

Other Investigators: Robert B. Bradley
Edwin E. Tucker
T. Phillip Pitner
Roderick E. Wasylishen (NRCC Fellow)

Cooperating Units: T. Clem (DRS-BEIB)
J. S. Cohen (NICHD)
J. A. Ferretti (DCRT)
H. T. Miles (NIAMDD-LMB)
C. N. R. Rao (PL-480 project, Indian Institute of Technology - Kanpur
V. Colburn, L. Barden (DCRT)
R. L. Lichter (City University of New York)

Man Years

Total:	5.5
Professional:	3.5
Other:	2

Project Description:

Objectives:

1. Elucidation of the forces within and between molecules, especially those of potential biological importance.
2. Development of spectroscopic methods for studying molecular structure and analyzing materials of chemical and biological interest.

Methods Employed:

The primary techniques used in this work are infrared spectroscopy (IR), Raman spectroscopy, and nuclear magnetic resonance spectroscopy (NMR). The three methods supplement each other in providing detailed information about the structure of molecules. In addition, the spectra are highly

sensitive to the effects of molecular interactions. For studies of hydrogen bonding, high precision vapor pressure measurements provide information complementary to that obtained by the spectroscopic techniques. Digital computer methods are used extensively.

Major Findings:

An examination of the theroetical basis for the new NMR method of rapid scan Fourier transform (RSFT) spectroscopy, as well as practical aspects of its implementation, has been published. This technique provides a valuable adjunct to pulse-FT NMR methods. The RSFT method can readily be implemented, with a suitable minicomputer, on commonly available continuous wave NMR spectrometers. Recent work indicates that it is especially useful in the study of weak proton NMR signals in the presence of a large solvent signal (e.g., H_2O). (Gupta, Ferretti, Becker)

The use of ^{13}C NMR spin-lattice relaxation times (T_1) to characterize hydrogen bonded species in t-butanol and in phenol has been explored. The values of T_1 increase markedly (a factor of six in one case) on dilution of the hydrogen bonded substance in an inert solvent. With more extensive data it should be possible to relate the T_1 behavior to the detailed molecular structures of the hydrogen bonded species. (Tucker, Becker)

The geometrical dependence of ^{13}C-^{15}N NMR coupling constants has been explored both theoretically (by the use of finite perturbation theory in the INDO approximation) and experimentally. The lone pair of electrons on the nitrogen atom plays a major role in determining the sign and magnitude of the coupling. (Wasylishen, Lichter)

Spin-lattice relaxation times (T_1) of the protons in imidazole have been found to be highly dependent on the presence of trace amounts of paramagnetic metal ions (probably Cu^{++}) present as minute impurities. The values of T_1 exhibit a pronounced variation with pH, since metal complexation is pH-dependent. Such a pH dependence in histidine-containing proteins might be misinterpreted (and perhaps has been, in published literature) as a pH-dependent conformational change in the secondary structure of the protein. (Wasylishen, Cohen)

An infrared and NMR investigation of hydrogen bonding in the highly hindered alcohol 2,4-dimethyl-3-ethyl-3-pentanol has been carried out to obtain reliable values for the equilibrium constant and enthalpy of dimer formation. (Tucker, Becker, Rao)

Both proton and ^{13}C NMR studies have been made of a series of substituted isothiazoles. The effect of substituents on the ^{13}C chemical shifts has been related to π electron density distributions, as calculated by the CNDO/2 method. (Wasylishen, Becker)

Significance to Biomedical Research and the Program of the Institute:

In this project we are attempting to obtain basic information on the structure and molecular interactions of a variety of important substances and to learn more about the nature of hydrogen bonding. Such information may aid in an understanding of the relation between structure and function of nucleic acids, enzymes and other biopolymers. In addition, the development of spectroscopic techniques applicable to molecules of biological activity and interest, such as steroids, alkaloids, pigments, etc., aids in the structure elucidation of many natural and synthetic substances studied throughout NIH.

Proposed Course of Project:

The rapid scan correlation NMR method is being examined in more detail, and it will be implemented in simplified form on one or more minicomputers.

With the planned purchase of a new superconducting magnet, we shall considerably enhance our capabilities for ^{13}C NMR, and shall initiate work on the extension of our NMR capabilities to other nuclei, such as ^{2}H, ^{14}N and ^{15}N. In the ^{13}C field we expect to concentrate particularly on the measurement and interpretation of relaxation times for alcohols and metal complexes.

We plan to complete a long study of vibrational assignments and determination of intramolecular force fields in 1-methyluracil and in p-benzoquinone.

Publications:

Becker, E. D.: Recent advances in Fourier transform instrumentation. Ann. N. Y. Acad. Sci. 222: 724-732, 1973.

Lichter, R. L., Dorman, D. E., and Wasylishen, R.: Geometrical dependence of carbon-nitrogen coupling constants. Oximes. J. Am. Chem. Soc. 96: 930-932, 1974.

Sharpless, N. E., Ferretti, J. A., and Bradley, R. B.: The nuclear magnetic resonance of heterocyclic compounds related to anthracene. Org. Magn. Reson. 6: 115-120, 1974.

Gupta, R. K., Pitner, T. P., and Wasylishen, R.: Fourier transform NMR of exchanging chemical systems. J. Magn. Reson. In press.

Gupta, R. K., Ferretti, J. A., and Becker, E. D.: Rapid scan Fourier transform NMR spectroscopy. J. Magn. Reson. In press.

Tucker, E. E., and Lippert, E.: High resolution nuclear magnetic resonance studies of hydrogen bonding. In Zundel, G., Schuster, P., and Sandorfy, C. (eds.): Recent Advances in Hydrogen Bonding Studies. Amsterdam, The Netherlands, North-Holland Publishing Company. In press.

REPORTED IN PRESS - 1973

Tucker, E. E., and Becker, E. D.: Alcohol association studies, Part II. Vapor pressure, 220 MHz proton magnetic resonance and infrared investigations of tert-butyl alcohol association in hexadecane. J. Phys. Chem. 77: 1783-1795, 1973.

Becker, E. D.: Nuclei other than hydrogen: Some nuclear properties and a discussion of their mechanisms. In Axenrod, T., and Webb, G. A. (Eds.): Nuclear Magnetic Resonance Spectroscopy of Nuclei Other Than Protons. New York, John Wiley and Sons, Inc., 1974, pp. 1-15.

Becker, E. D.: Introduction to pulse and Fourier transform methods. In Axenrod, T., and Webb, G. A. (Eds.): Nuclear Magnetic Resonance Spectroscopy of Nuclei Other Than Protons. New York, John Wiley and Sons, Inc., 1974, pp.17-29.

Serial No. NIAMDD-LCP-6
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Molecular Dynamics

Previous Serial Number: NIAMDD-LCP-7

Principal Investigator: Ira W. Levin

Other Investigators: Robert A. R. Pearce
Robert C. Spiker, Jr. (NIH Research Fellow)

Cooperating Units: S. Abramowitz, National Bureau of Standards
William C. Harris, Furman University, Greenville, South Carolina
N. Gershfeld, NIAMDD-LPB

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

An understanding of the vibrational characteristics of moderate to large molecular systems through analyses of Raman and infrared spectra, the development and determination of reliable model intramolecular force fields and the interpretation of molecular charge distribution (infrared and Raman intensity) parameters.

Methods Employed:

Various laser Raman and infrared spectroscopic procedures provided the required data for the vibrational analyses. The intramolecular force field computations were necessarily performed on a large digital computer.

Major Findings:

1. Although far infrared and microwave studies provide estimates for various long range potential energy coupling terms between the methyl

rotors in $(CH_3)_2X$ systems, relatively little information exists on the significance and magnitude of the methyl-methyl interaction parameters between vibrations other than the torsional modes. In order to assess the relative importance of additional potential constants introduced through intermethyl group coupling effects, intramolecular force fields were developed specifically for the dimethyl sulfide system. Raman spectra of both polycrystalline and dilute solid solutions of dimethyl sulfide-d_0 and dimethyl sulfide-d_6 were observed at liquid nitrogen temperatures ($\sim$ 77°K) using argon ion laser excitation. Mixed isotopic crystals of the unique molecular species were prepared in molar ratios of 3/100. The solid solution frequency data for both isotopic molecules provided the basis for a detailed vibrational assignment from which various model internal valence force fields were developed. The final 14 parameter force field, representative of the vibrations of dimethyl sulfide in a static crystal field, emphasizes the contribution to the potential function of a small, but significant, methyl-methyl coupling term between the anti H-C-S angular vibrational displacement coordinates. (Levin, Pearce and Spiker)

2. Although considerable efforts have been directed toward the elucidation of the bonding of oxygen in heme proteins, the structure of the iron dioxygen complex is not well understood. The only direct structural evidence reported in the literature is a vibrational study of the oxyhemoglobin system (Caughey et al. Biochem. Biophys. Res. Commun. 55, 91 (1973)) in which the interpretation is based upon vibrational frequencies of model dioxygen species containing metals other than iron. The conclusion of these authors was that a non-linear end-on bonding exists for O_2 to Fe. Since no distinct iron dioxygen system has been reported, we prepared a model FeO_2 complex by reacting molecular beams of iron and oxygen at 1700°K and trapping the products in an argon matrix at 12°K. Several classes of iron-oxygen products appeared. On the basis of $^{16}O_2$, $^{18}O_2$ and ^{16}O-^{18}O studies, however, we clearly identified a symmetrical isosceles FeO_2 species as opposed to the alternate structure with oxygen bound end-on to the iron. (Levin and Abramowitz)

3. Laser Raman spectroscopic techniques were used to investigate conformational changes in lecithin multilayer and vesicle bilayer model membrane systems. In particular the melting processes of the paraffin side chains in 1,2 dipalymitoyl phosphatidyl choline (DPL) and 1,2 dimyristoyl phosphatidyl choline (DML) were followed by monitoring either the C-H stretching or C-C stretching regions of the Raman spectrum. The studies to date have progressed as follows: (1) A relatively detailed assignment of 1,2 dipalymitoyl phosphatidyl choline has been proposed with a particular emphasis on sorting the phosphate and choline C-N modes from the hydrocarbon distortions. (Levin and Spiker) (2) Precise frequency shift measurements of DPL and DML in the C-C stretching region (1060 - 1130 cm^{-1}) were used (a) to locate the gel-liquid crystalline transition temperatures T_c and (b) to probe the changes occurring in chain fluidity, above and below T_c, when other components, such as cholesterol, were added to the

system. (Levin and Spiker) (3) Changes in spectral intensities of the DPL vesicle bilayer systems as a function of temperature provided values for the energy differences ΔH between gauche-type and trans-type forms of the hydrocarbon chains. Specific systems investigated were DPL-H_2O, DPL (anhydrous), DPL-cholesterol and DPL-cholesterol-H_2O dispersions. These measurements suggest that several states of aggegation exist between the various components of the DPL-cholesterol-H_2O system. (Levin, Spiker, Gershfeld)

4. Raman spectra of liquid and polycrystalline quadricyclene [quadricyclo $(2.2.1.0^{2,6}0^{3,5})$ heptane] were studied from 100 to 3200 cm^{-1}, while the infrared absorption spectra were investigated, from 400 to 3200 cm^{-1}. A vibrational assignment for this highly strained ring system was proposed on the basis of observed band positions, relative intensities, depolarization ratios and normal coordinate analyses of related molecules. We attempted to assess both the influence of strain and vibrational couplings upon the observed spectra and to establish what may be considered "characteristic frequencies" for skeletal modes of highly strained ring molecules. (Levin and Harris)

Significance to Biomedical Research and the Program of the Institute:

Since experimentally determined vibrational frequencies and charge distribution parameters reflect the sum of several subtle electronic and nuclear contributions, force field determinations permit both quantitative and qualitative descriptions of the dominant terms and create a link between empirical information and bond properties.

Proposed Course of Project:

1. Development of modified internal valence force fields (a) for interpreting the spectra of moderately complex molecules in terms of dominant kinetic and potential energy coupling effects and (b) for calculating molecular structures from transferable potention function information.
2. Investigation of the vibrational behavior of multilamellar and vesicle model membrane systems, with an emphasis on both the gel and liquid crystal phases of various lecithin-water dispersions. As the experimental techniques are refined and the Raman spectra are better understood, normal coordinate calculations will be applied toward establishing specific conformational changes in phospholipid membrane components.

Publications:

Levin, I. W., Pearce, R. A. R., and Harris, W. C.: Vibrational spectra and normal coordinate analysis for trans-2-butene-d_0 and trans-2-butene-d_8. J. Chem. Phys. 59: 3048-3061, 1973.

Levin, I. W., and Pearce, R. A. R.: Vibrational analysis of the Raman spectra of polycrystalline cis-2-butene. J. Mol. Spectrosc. 49: 91-99, 1974.

Pearce, R. A. R., and Levin, I. W.: Vibrational coupling effects and potential functions for cis- and trans-1,2-difluorodiimide and cis- and trans-1,2-difluoroethylene. J. Chem. Phys. 59: 2698-2710, 1973.

REPORTED IN PRESS - 1973

Harris, W. C., and Levin, I. W.: Low temperature Raman spectroscopy of molecular solids. In Mathieu, J. P. (Ed.): Advances in Laser Raman Spectroscopy, Vol. I. London, Heyden and Son, Ltd., 1973, pp. 597-610.

Levin, I. W., and Harris, W. C.: Interpretation of the vibrational spectra of bicyclic hydrocarbons: norbornane and norbornadiene. Spectrochim. Acta 29A: 1815-1834, 1973.

Pearce, R. A. R., Levin, I. W., and Harris, W. C.: Raman spectra and vibrational potential function of polycrystalline azomethane and azomethane-d_6. J. Chem. Phys. 59: 1209-1216, 1973.

Serial No. NIAMDD-LCP-7
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The physical chemistry of membranes and complex membrane systems of biological interest

Previous Serial Number: NIAMDD-LCP-1

Principal Investigator: Gerald M. Shean

Other Investigators: None

Cooperating Units: Karl Sollner (Consultant)

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

The physico-chemical study of membranes, and complex membrane systems and models with the ultimate aim of providing a rational basis for various complex membrane phenomena in living systems.

Methods Employed:

Conventional chemical-analytical and electrochemical methods, especially potentiometric measurements as reported previously.

Major Findings:

Continuation of experimental work with liquid cation exchanger membranes showed that the .1 N K^+ vs. 1 N Na^+ bi-ionic potentials (BIPs) across membranes consisting of dipicryl amine (DPA), 2,2', 4,4', 6,6' hexanitrodiphenyl amine in o-nitrotoluene are essentially the same regardless of how much DPA is in the membrane. The bi-ionic potentials of membranes with DPA concentrations of .02 M., .002M., .002 M and .00002 M were 108.9 mv, 108.1 mv, 108.1 mv and 107.4 mv respectively with the more permeable potassium ion impressing its positive charge on the sodium side of the membrane.

Various solvents were used in the .02 M DPA membranes to determine how they would affect the BIPs (and therefore the specificities). The highest BIP with .1 N K^+ vs. .1 N Na^+ was found with o-nitrotoluene which indicated almost a one hundred fold preference of potassium ion over sodium ion. The smaller BIPs with most other solvent-DPA mixtures were due to a combination of less potassium specificity and leakage of anions across the membrane. Some solvent-DPA mixture membranes even reversed the usual order of ion preference and preferred the sodium ion over the potassium ion--the potassium side of the BIP cell was positive. There was no correlation between potassium preference and whether the solvent was an electron donor or hydrogen bonding molecule.

Preliminary experiments indicate that fatty amide membranes may be of value in studying osmosis across oil membranes.

Significance to Biomedical Research and the Program of the Institute:

Movement of solvent and solutes is an important aspect of all life processes. This project tries to provide solidly founded, nonspeculative physicochemical background information on the functional behavior of membranes in general. At the same time, these membrane studies have furnished a new, now widely used tool for the electrometric determination of ion activities - the use of permselective membranes as membrane electrodes. It is perhaps also of interest that Sollner's "permselective" membranes are the prototypes of the ion exchange membranes which are used now in the electrodialytic desalination of sea and brackish water.

Proposed Course of the Project:

Investigating the use of liquid anion and cation exchanger membranes in cells that will accumulate both anions and cations against their concentration gradients. These "accumulation" cells would be "driven" by other anions and cations and the accumulation would be the result of Donnan equilibria across the membranes. Searching for suitable membrane materials to study osmosis across oil membranes.

Publications:

Caplan, S. R. and Sollner, K.: The influence of the characteristics of the activating polyelectrolyte in the preparation and on the properties of interpolymer ion exchange membranes. I. The rational principles of membrane preparation and their experimental test. J. Colloid. Interface Sci. 46: 46-66, 1974.

Caplan, S. R. and Sollner, K.: The influence of the characteristics of the activating polyelectrolyte in the preparation and on the properties of interpolymer ion exchange membranes. II. Further experimental studies on the sorption, desorption, and permeation of polyelectrolyte in collodion membranes of high porosity. J. Colloid Interface Sci. 46: 67-76, 1974.

Caplan, S. R. and Sollner, K.: The influence of the characteristics of the activating polyelectrolyte in the preparation and on the properties of interpolymer ion exchange membranes. III. Some considerations concerning peculiarities of the functional properties of polyelectrolyte activated "internal swelling" membranes. J. Colloid Interface Sci. 46: 77-84, 1974.

Tamamushi, R.: An experimental study of Gibbs-Donnan membrane equilibria across permselective membranes which involve the ions of strong inorganic electrolytes. Bull. Chem. Soc. Jap. 46: 2701-2708, 1973.

Sollner, K.: An outline of the electrochemistry of porous membranes. J. Dent. Res. 53: 267-279, 1974.

Sollner, K.: Prologue in the form of a personal letter to Karl Kammermeyer. In Membrane Separation Processes, Kammermeyer, K. (ed.). Interscience-Wiley. In press.

Serial No. NIAMDD-LCP-8
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Chemistry and biosynthesis of natural compounds, and instrumental methods used in their study.

Previous Serial Number: Same

Principal Investigator: Ulrich Weiss

Other Investigators: Kenner C. Rice
Robert J. J. Ch. Lousberg

Cooperating Units: Dr. Roderick Wasylishen (NIAMDD-LCP)
Dr. Norman E. Sharpless (NIAMDD-LCP)
Dr. Robert J. Highet (NHLI-LC)
Dr. J. V. Silverton (NHLI-LC)
Dr. T. Akiyama (NHLI-LC)

Man Years:

Total:	2.25
Professional:	2.25
Other:	0

Project Description:

Objectives:

Isolation in pure form, and elucidation of the chemical constitution of a variety of naturally occurring substances of chemical, biosynthetic, or biological interest. Study of instrumental techniques used in such work. Corroboration of chemical structure by total synthesis. Synthesis of compounds of medical or biological interest.

Methods Employed:

Standard procedures of organic chemistry, and instrumental methods (UV, IR, NMR spectroscopy). For measurement of optical rotatory dispersion and circular dichroism, the Cary 60 spectropolarimeter and its CD attachment are used.

Major Findings:

Work has been initiated on the synthesis of certain derivatives of morphine and codeine which embody modifications of the parent molecules that had not been achieved previously. Of the desired new compounds, 1-fluorocodeine has been obtained so far (R. Lousberg); preparation of 2-fluorocodeine is in progress. It is hoped that pharmacological study of this compound, and of others to be made, will contribute to the understanding of the mechanism of analgetic action, and of the way in which these drugs bind to the opiate receptor protein present in the central nervous system.

Study of the reaction of dimethyl β-ketoglutarate and glyoxal at room temperature in aqueous solution has been continued. In particular, the influence of pH on the nature of the compounds has been examined; better control of this variable has been achieved by working in phosphate-citrate buffers at pH 3, 4, 5, and 6. At pH 6 (and to a lesser extent at pH 5), a new, very insoluble β-keto ester crystallized out in high yield. Treatment with acid removed four of the six carbomethoxy groups and yielded a compound $C_{17}H_{20}O_8$, whose nmr spectrum indicated a very non-symmetrical, complex structure; in contrast, the two compounds obtained previously at more acidic pH were symmetrically constituted. The structure of the C_{17} compound was established by x-ray crystallography (Drs. T. Akiyama and J. V. Silverton); it is dimethyl 1,10-dihydroxy-tetracyclo [6.5.0.$0^{2,10}$.$0^{3,7}$] tridecan-5,12-dione 2,8-dicarboxylate--a structure whose complexity is well indicated by its name. The precursor of this C_{17} compound has the same ring system with additional carbomethoxy groups in positions 4, 6, 11, and 13. Formation of a ring system as complicated as this in one step from readily available starting materials under "physiological conditions" (aqueous solution, pH close to neutrality, room temperature) must be almost unprecedented. The aqueous filtrates, on acid-catalyzed saponification, yielded the two compounds obtained previously at pH 3-5: bicyclo [3.3.0] octan-3,7-dione and endo-tetracyclo [5.5.1.$0^{2,6}$.$0^{10,13}$] tridecane-4,8,12-trione (see Report for 1973) together with small amounts of the new exo-stereoisomer of the latter. The structure assigned to the last-named compound rests in part upon comparative study of the dipole moments and ^{13}Cmr spectra of the two isomers by Drs. Sharpless and Highet, respectively.

Work on the synthesis of a nitroxyl radical suitable for assay of Ca^{++} in biological materials (see Report for 1973) has been continued. Many approaches have been tried and many new intermediates were obtained, but the desired compound has proved elusive. Another compound requested by Dr. William Hagins, the nitroxyl radical of N-succinoyl-2,2,6,6-tetramethyl-4-aminopiperidine has been synthesized from the aminopiperidine nitroxyl and succinic acid. It acts as a quencher of fluorescence in biological materials.

The conformational problem presented by hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one (see Report for 1973) has been pursued further in

cooperation with Dr. Wasylishen. Various model compounds were synthesized. Nmr studies have confirmed the previously observed very high coalescence temperature of the oxazepinone and of two of its derivatives; the energy barriers to conformational inversion calculated from these temperatures had the exceptionally high values of ∿ 20±1 kcal/mole. From study of model compounds, this barrier appears to be due to t' presence of both the hexahydro-1,2-oxazepine ring and the four methyl groups.

The pharmacological action of hexahydro-3,3,7,7-tetramethyl-1,2-oxazepine-5-one on the mouse has been briefly examined by Dr. Zdenka Horákova (NHLI-ET) and found similar to that on the frog reported in 1897. Further study of this action is being undertaken by Prof. Ulf S. von Euler at the Karolinska Institute, Stockholm.

Significance to Biomedical Research and the Program of the Institute:

The 1- and 2-fluoro derivatives of morphine and codeine should give information on the mechanism of action of analgesic drugs. Replacement of the hydrogen atom at either of these positions has always been found to decrease analgesic action. This effect could be caused by the space-requirement of the substituents examined so far, all of which are substantially larger than a hydrogen atom; because of this, they might interfere with the proper attachment of the molecule to some receptor surface. Alternatively, the effect of the substituents might be based upon their electronic influence upon the aromatic ring to which they are attached. Of possible substituents, fluorine alone is hardly larger than a hydrogen atom; its steric influence should thus be negligible, while its electronic effect is powerful. The study of the fluorinated derivatives should thus permit an evaluation of the relative importance of steric vs. electronic influences. It seems possible that the former is the more important one, in which case the fluoro compounds might retain analgetic activity, perhaps in a qualitatively modified form. It is impossible to predict whether this modification of activity would be favorable or not, but the chances of finding improved drugs is at least not excluded; introduction of fluorine has several times in the past led to improved drugs.

In the formation of complex polycyclic compounds from dimethyl β-ketoglutarate and α-dicarbonyl compounds such as glyoxal under so-called "physiological conditions," two types or reactions must take place: aldol condensations and Michael additions; it is not possible to explain the structures of the products in any way except through sequences involving these reactions. Both, but particularly the first-named one, are among the most important reactions occurring in the biosynthesis of natural compounds. The reactions studied by us are thus of great interest as model reactions for biochemical events, especially in view of the complicated non-symmetrical structure of one of the products which is strongly reminiscent of the structures of some natural products.

In addition, the compounds readily obtained by us, and many analogous ones potentially accessible through variations of the starting materials, might well lend themselves to transformation into medicinally interesting substances. Very many such variations can be devised easily.

The pharmacological effect of hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-dione consists in a perfectly reversible paralysis, especially of the extremities, not accompanied by any detectable influence upon respiration or heart. This unusual type of activity prompted Prof. von Euler to request spontaneously a sample for study in his institute. The strong preference for one of the many possible conformations is of interest in relation to the pharmacological action of the compound.

Proposed Course of Project:

In addition to preparation of the fluorinated codeine and morphine derivatives, it is planned to explore possibilities for introduction of substituents into positions 15 and 16. Few compounds modified in these positions have ever been made. Again, the effect of such modifications seems quite unpredictable, but study of the compounds should, at least, contribute to our knowledge of structure-activity relationships in the morphine series and might, at best, yield useful drugs. Further cooperation with Dr. Lousberg, after his return to Utrecht, is planned, and similar cooperation with Drs. K. C. Rice and Everette L. May might be possible. Dr. Werner Klee (NIMH-LGCB) has expressed his interest in studying the binding of our new morphine derivatives to the opiate receptor protein from the central nervous system, and Dr. Robert Highet wishes to include the fluoro-compounds in his nmr studies of fluorinated compounds and their biological transformations.

The extent to which work on the hexahydrooxazepinone should be continued will depend upon Prof. von Euler's findings. If they indicate any pharmacological interest, the various related compounds already available should be studied, and the generality of formation of hexahydro-1,2-oxazepin-5-ones by addition of hydroxylamine to cross-conjugated ketones other than phorone should be examined. Unpublished work in the laboratory of Dr. J. T. Davis, McGill University, Montreal, has shown that one ketone of this class, santonin, indeed adds hydroxylamine in an entirely analogous fashion. Similar reactions of compounds such as prednisone, which belongs to the same group of substances, might well deserve exploration.

Our work on reaction of dimethyl β-ketoglutarate with glyoxal should be capable of being extended to many analogous reactions by using other β-keto esters and α-dicarbonyl compounds; innumerable further transformations of the very reactive substances potentially obtainable suggest themselves. Dr. James M. Cook, Chemistry Department, University of Wisconsin at Milwaukee, has expressed great interest in exploring those possibilities with his students, and arrangements for cooperative work have been made.

The biosynthesis of the 9-phenylphenalenone from Lachnanthes (see Report for 1973) is to be studied further in cooperation with Dr. J. M. Edwards. Studies on the chiroptical properties of non-planar conjugated dienes are to be resumed.

Publications:

Edwards, J. M., Qureshi, I. H., Weiss, U., Akiyama, T., and Silverton, J. M.: A two-step synthesis of a triketone of the endo-tetracyclo [5.5.1.$0^{2,6}$.$0^{10,13}$] tridecane series. X-ray crystallographic proof of the structure and stereochemistry. J. Org. Chem. 38: 2919-2920, 1973.

Edwards, J. M., and Weiss, U.: Root pigments of Lachnanthes tinctoria. Phytochemistry. In press.

Weiss, U., Tillack, J. V., and Kennard, C. H. L.: Stereochemistry of dihydrometacodeinone: x-ray crystallographic proof of the configuration and conformation derived from the NMR spectrum. Proc. Roy. Aust. Chem. Inst. In press.

REPORTED IN PRESS - 1973

Rice, K. C., and Weiss, U.: Identification of the so-called triacetonehydroxylamine as hexahydro-3,3,7,7-tetramethyl-1,2-oxazepin-5-one. Tetrahedron Letters: 1615-1618, 1973.

Serial No. NIAMDD-LCP-9
1. Chemical Physics
2. Molecular Biophysics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Titles:

1. A study of photoreactions and the mechanisms by which these reactions occur.
2. An investigation of the absolute stereochemistry of several dihydro diols obtained from microbial oxidation of aromatic substrates.
3. New methods for the asymmetric synthesis of cyclic alcohols and diols.

Previous Serial Number: Same

Principal Investigator: Herman Ziffer

Other Investigators: Jeffrey I. Seeman

Cooperating Units: Donald M. Jerina (NIAMDD-LC)
Herman J. C. Yeh (NIAMDD-LC)
D. T. Gibson, University of Texas
Robert J. Highet (NHLI-IR-CH)

Man Years:

Total:	1.25
Professional:	1.25
Other:	0

Project Description:

Objectives:

1. To examine the photochemistry of a number of β,γ-unsaturated ketones in order to learn more about the mechanism and stereochemistry of their reactions.
2. To determine the absolute stereochemistry of several dihydro diols, microbial metabolites of aromatic substrates, in order to learn more about the enzyme-substrate complex.
3. To develop methods for the asymmetric synthesis of several alcohols and diols of biological significance.

Methods Employed:

The Cary 60 spectropolarimeter has been used to obtain ORD and CD measurements. Standard organic chemical techniques were employed for the synthesis and proof of structure of a number of compounds. Along with the usual spectroscopic measurements (NMR, IR and UV) we have used the Varian time averaging computer in conjunction with our NMR equipment to obtain several important NMR measurements necessary for structure determinations.

Major Findings:

1. The proton and ^{13}C nmr characteristics of the 3-methyl cyclohexan-1,2-diols have been determined and the spectra have been analyzed. The results establish limits for correlating the stereochemistry of the substituents with chemical shift.
2. The relative and absolute stereochemistry of two trans p-mentha-2,3-diols have been determined and cross checked using a combination of spectral measurements. The methods demonstrate that the "Dibenzoate Chirality" rule can be used to determine the absolute stereochemistry of trans diols.
3. A study of the effect of α-methyl groups on the photochemistry of 3,4,5,6,7,8-hexahydronaphthalen-2(1H)-one has shown that the introduction of methyl groups to the α-carbon dramatically increases the importance of the singlet reaction relative to the triplet reaction. This is a consequence of an increase in the rate of α-cleavage relative to the rate of intersystem crossing.
4. The absolute stereochemistry of a microbiol metabolite of ethyl benzene has been determined as (+) cis-1(S), 2(R)-dihydroxy-3-ethyl cyclohexa-3,5-diene by spectroscopic methods. The absolute stereochemistry about the hydroxyl groups is identical to the analogous metabolite from toluene.

Significance to Biomedical Research and the Program of the Institute:

Information on the absolute stereochemistry of metabolites will assist in developing models which describe the way substrates attach to enzymes and the course of the reaction.

The increased knowledge derived from studies of the mode and mechanism of photochemical reactions permit a more rational use of photochemical reactions for the preparation of compounds that are difficult to prepare by other means.

Proposed Course of Projects:

1. To determine and compare the absolute stereochemistry of a number of cis dihydro diols produced by microbiol metabolites of aromatic substrates.

2. To prepare and examine the photochemistry of some 5α and 5β vinyl cholestan-4-ones to better understand the factors that influence α-cleavage and recombination in β,γ-unsaturated ketones.
3. To examine the photochemistry of selected cyclopropyl ketones to determine the multiplicity of the reactive species and the factors that direct ring closure of the diradical intermediate.
4. To examine the effects of substituents on the rates of isomerization about the carbon to nitrogen double bond.

Publications:

Engel, P. S., Schexnayder, M. A., Ziffer, H., and Seeman, J. I.: The effect of α-methyl groups on the photochemistry of 3,4,5,6,7,8-hexahydronaphthalen-2(1H)-one. J. Am. Chem. Soc. 96: 924-925, 1974.

Seeman, J. I., and Ziffer, H.: The stereochemistry of the oxa-di-π-methane rearrangement. Tetrahedron Letters: 4413-4416, 1973.

Seeman, J. I., and Ziffer, H.: The role of α-cleavage in the photochemistry of A-homo-4A-cholestene-3-one. Structure revision of photoproducts. Tetrahedron Letters: 4409-4412, 1973.

Bright, W. M., Langs, D. A., Silverton, J. V., Ziffer, H., and Eisner, U.: Tetraethyl 3,9-diazahexacyclo 6.4.0^{2,7}.0^{4,11}.0^{5,10})dodecane-1,5,7,11 tetracarboxylate, $C_{22}H_{30}N_2O_8$. Cryst. Struct. Comm. 3: 1-6, 1974.

REPORTED IN PRESS - 1973

Ziffer, H., Jerina, D. M., Gibson, D. T., and Kobal, V. M.: Absolute stereochemistry of the (+)-cis-1,2-dihydroxy-3-methylcyclohexa-3,5-diene produced from toluene by Pseudomonas putida. J. Am. Chem. Soc. 95: 4048-4049, 1973.

Serial No. NIAMDD-LCP-10
1. Chemical Physics
2. Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effects of altitude (hypoxia), exercise, and other environmental stresses on physiological, biochemical and pathological mechanisms in animals.

Previous Serial Number: Same

Principal Investigator: Paul D. Altland

Other Investigators: Mr. H. F. Brubach and Mr. M. G. Parker

Cooperating Units: None

Man Years

Total:	4.5
Professional:	2.5
Other:	2

Project Description:

Objectives:

To determine physiological, biochemical, and pathological changes occurring during exposure to hypoxia induced by decompression or by low oxygen concentrations in nitrogen. To determine the mechanism of action of cross adaptation between altitude acclimation and exercise in the cold. To determine the relationship between pathological changes and elevated serum enzyme concentrations in rabbits exposed to cold. To determine the effect of hypoxia upon the susceptibility of rats to experimental bacterial endocarditis.

Methods Employed:

Biochemical analysis of serum enzymes and metabolic end products. Physiological measurement of body temperature, exercise performance and hypoxic tolerance. Histopathological methods for demonstration of fatty and necrotic changes in vital organs. Operation of two altitude chambers and an animal exerciser at 1.7°C.

Major Findings:

In experiments designed to determine the possible relationship of serum enzyme and tissue changes in shaven rabbits exposed to cold (-5°C) it was found that severely hypothermic rabbits developed marked elevations in

serum concentrations of glutamic oxalacetic transaminase, glutamic pyruvic transaminase, aldolase, creatine phosphotransferase and lactic dehydrogenase within 10 to 16 hours. Serum enzyme concentrations reached peak levels within 1 day after removal from the cold and returned to normal within 6 days. Serum levels of isoenzyme lactic dehydrogenase five ($SLDH_5$) greatly increased and $SLDH_1$ decreased in severely hypothermic rabbits. This indicated that striated muscle tissue contributed to serum concentrations of SLDH since $SLDH_5$ is abundant in muscle. About one-half of the rabbits exposed to cold showed foci of cardiac necrosis, multiple pulmonary hemorrhages, and fatty changes in the liver, kidney and striated muscles. It is apparent that serum enzymes reached peak concentrations within the first day after cold exposure at a time when fatty changes and necrosis were found in the heart and other organs. The tissue changes were most frequent and severe in the hypothermic rabbits with the highest serum enzyme concentrations. Within 3 to 6 days fatty changes had largely disappeared, but the myocardial lesions persisted in the form of granulation and scar tissue.

Significance to Biomedical Research and the Program of the Institute:

This investigation shows that analysis of serum enzymes of individuals exposed to severe cold may be used as an aid in the diagnosis of the severity of pathological changes. In cases of extreme hypothermia serum enzymes may be correlated with fatty changes in the heart, liver, kidney and striated muscles and with cardiac necrosis. It is important to make the serum enzyme determination immediately after the cold exposure and at daily intervals thereafter in order to avoid overlooking early serum enzyme changes. Serum enzyme changes usually persist for only about 24 hours.

Proposed Course of Project:

Experiments will be conducted on the effects of cross adaptation between altitude and exercise at room temperature and in the cold. Investigations will be continued on the effects of exercise and hypoxia on the susceptibility of rats to experimental bacterial endocarditis.

Publications:

Altland, P. D., Highman, B., and Sellner, R. G. (with the technical assistance of M. G. Parker): Serum enzyme and tissue changes in shaven rabbits exposed to cold. Cryobiol. (in press).

REPORTED IN PRESS - 1973

Altland, P. D., Highman, B., and Sellner, R. G. (with technical assistance of M. G. Parker and H. F. Brubach): Tolerance of cold-acclimated and unacclimated rats to hypoxia at 1.7°C. Int. J. Biometeor. 17: 59-71, 1973.

Serial No. NIAMDD-LCP-11
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Molecular structure, organization and intermolecular forces

Previous Serial Number: Same

Principal Investigator: Elliot Charney

Other Investigators: Joan Rosenfield

Cooperating Units: Dr. Daniel Glaubiger, Stanford University Medical School
Dr. Kurt Kohn, NCI

Man Years:

Total:	3.5
Professional:	2.0
Other:	1.5

Project Description:

Objectives:

The general objectives of this research are to determine the role of molecular structure and of intermolecular forces in chemical, physical and biological phenomena by exploring the optical properties of molecules (spectroscopic absorption, optical rotatory dispersion, circular and linear electric-field induced dichroism).

Methods Employed:

All of the methods employed, some of which are unique to this laboratory, have been described in previous reports.

Major Findings:

1. Electric field induced properties of large molecules

   a. DNA: New data obtained on highly monodispersed samples of sonicated DNA confirm our earlier work which demonstrated that the optical properties high molecular weight DNA in solution are not those predicted from a Watson-Crick-Wilkins structure and the optical properties of the isolated nucleic acid bases.

The significance of the new data is that it is obtained from samples known to be nearly rigid rods even in the absence of the electric field used in these experiments to orient them, and as a consequence, there is no ambiguity arising from disorientation due to flexibility.

b. Polyriboseadenylic acid: Calculations of the effect of strong electric fields on two dipoles with a universal hinge between them, having the approximate dipole moment and mass of two helical terms of DNA and a reasonable force constant for bending at the hinge, have enabled us to predict that in fields of about 10,000 volts/cm flexible macromolecules such as DNA and Poly A should become essentially rod-like. These are field strengths at which these molecules are virtually completely oriented with their long axis in the field direction. New electric dichroism experiments on shorter, more rigid molecules of double stranded poly A have provided beautiful confirmation of this; the dichroism of the Poly A of M_w = 123,000 is the same as that of the more flexible poly A of M_w = 500,000 when both are fully oriented by the electric field.

Furthermore, dichroism relaxation data indicate that the low molecular weight poly A is slightly more flexible than DNA of the same molecular weight, while the higher molecular weight poly A is much more flexible than DNA of the same molecular weight. This raises a question of whether the role of long stretches of poly A in some RNA is to introduce physical flexibility. No role has as yet been assigned to these poly A stretches.

c. Binding of antibiotics to DNA (with Dr. Glaubiger and Dr. Kohn)

A manuscript reporting on the work done last year has been drafted and is being prepared for publication.

Significance to Biomedical Research and the Program of the Institute:

Optical and electrical properties of small and large molecules helps elucidate their structures and the relationships between structure, reactivity, and the intermolecular forces.

Proposed Course of Project:

1. An attempt will be made to determine from electric dichroism data what structural perturbations if any are produced in DNA by environments which have profound affects on biological phenomena associated with DNA, in particular the effect of glycerol which has been shown to stimulate total RNA synthesis and act as a replacement for cyclic

AMP and cyclic AMP receptor in bacterial transcription.

2. The project underway at the end of last year to measure the linear dichroism of DNA fibres which can also be studied by X-ray, designed to settle the structural question arising from the electro optical investigation, was temporarily suspended to permit Dr. Hofrichter to complete a very timely investigation of the orientation of haemoglobin S in a sickled cell. The DNA fibre project has just been resumed and is expected to be completed.

3. Optical activity of α-diketones (with Dr. Rosenfield)

   Since the publication of our work [Charney and Tsai, J. Amer. Chem. Soc. 93: 7123-7132, 1971] on the lower excited states of α-diketones new studies have been undertaken in laboratories at the Universities of Virginia and Kansas and Louisiana State University, Cornell University and at the State University of New York at Stony Brook. These studies, some of which are theoretical and some experimental, have reopened the question of the interpretation of these states. We are preparing to examine, by one of the best quantum mechanical methods available, the optical activity of these states with a view to determining which of several different interpretations is most plausible and in the process to examine questions relating to the validity of this approach for calculating optical activity of $n\pi^*$ states.

Publications:

Charney, E.: Electro-optics in the infrared region. In O'Konski, C. T. (ed.): Molecular Electro- Optics. New York, New York, Marcel Dekker. In press.

Charney, E. and Milstien, J. B.: Electric dichroism of polyriboseadenylic acid. Biopolymers. In press.

Serial No. NIAMDD-LCP-12
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Conformation and electronic structure of biological molecules

Previous Serial Number: Same

Principal Investigator: William A. Eaton

Other Investigators: Louise K. Hanson
James Hofrichter

Cooperating Units: Dr. Philip D. Ross, NIAMDD-LMB
Dr. Philip J. Stephens (Univ. of Southern California)
Dr. Marvin W. Makinen, formerly NIDR-LB
Dr. Martha L. Ludwig (Univ. of Michigan)
Dr. Eduardo A. Padlan, NIAMDD-LMB
Dr. Takashi Yonetani (Univ. of Pennsylvania)

Man Years:

Total:	3.2
Professional:	2.7
Other:	0.5

Project Description:

Objectives:

The general aim of the research is to relate the stereochemistry and electronic structure of biological molecules, particularly proteins, to their biochemical reactivity and function. From our investigation of the structure of hemoglobin S fibers and the kinetics and thermodynamics of gelation we hope to elucidate the detailed molecular mechanism of sickling.

Methods Employed:

In addition to conventional biochemical and spectroscopic methods, techniques have been developed for measuring polarized absorption and other anisotropic optical properties of microscopic samples, such as single red cells and microcrystals, from the ultraviolet to the near infrared wavelength regions.

Major Findings:

1. **Sickle cell anemia**

   a. **Structure of hemoglobin S fibers (with Dr. Hofrichter)**

   We have completed a study on the molecular orientation in hemoglobin S fibers from polarized absorption measurements on single sickled red cells using our microspectrophotometer. Previous spectroscopic studies on heme protein single crystals have provided the basis for this investigation by characterizing both experimentally and theoretically the detailed absorption properties of the heme chromophore in plane-polarized light. The polarization ratio of single sickled red cells gives a lower limit for that of a single hemoglobin S fiber. Coupled with the absorption properties of the deoxyhemoglobin A molecule, the single-cell results restrict the orientation of the long (x) molecular axis to within 22° of the fiber axis. Adopting the stacked-ring model of Finch and co-workers for the molecular positions and the additional constraint that at least one mutated (β6) site is part of an intermolecular contact, model building studies on the computer show that three types of models for the intermolecular contacts in the fiber are permitted by our optical results. In all three models the true molecular dyad (y) axis passes through some part of an adjacent molecule in the same ring. This range of orientations for the y axis is approximately perpendicular to those described in previous models and places at least one β6 residue in position to be part of a contact between molecules in the same ring.

   b. Kinetics and thermodynamics of hemoglobin S gelation (with Drs. Hofrichter and Ross)

   Deoxyhemoglobin S in concentrated solutions aggregates and forms an ordered, birefringent phase - the so-called gel. It is this gel which distorts red cells upon deoxygenation. This system exhibits a temperature-induced phase transition which we have begun to characterize by measuring the heat capacity, viscosity, optical birefringence, and distribution of hemoglobin between the gel and liquid phases. The enthalpy change for the transition, as measured in a scanning microcalorimeter, is only 1200 - 2700 calories/mole hemoglobin. This small magnitude is suggestive of a process involving weak intermolecular interactions and small conformational changes. This is an important result for model building studies in which it has been assumed that deoxyhemoglobin S does not change conformation upon aggregation into fibers.

Complete melting curves have been obtained from measurements of the optical birefringence versus temperature. There is considerable hysteresis as a result of the relatively slow rates of gelation compared to degelation. The midpoint of the melting curve is an excellent measure of the thermodynamic tendancy of hemoglobin S to gel and since only 5 microliter samples are required for the measurement this technique should provide a useful assay for screening and investigating anti-sickling agents.

We are investigating the kinetics of gelation and degelation by temperature-jump experiments. Both gelation and degelation exhibit several distinct kinetic steps. In sequential temperature-jump experiments along the melting curve, the degelation process may be formally described by two first-order processes with at least a ten-fold difference in rate constants. One explanation for the two observed rates is that the dissolution of the gel phase into monomers is associated with the fast process, while the slow process corresponds to the disorientation of fibers.

2. Heme proteins

a. Electronic structure of heme in heme proteins (with Drs. Stephens and Makinen)

Most of the experimental work on the investigation of the optical spectra of a wide variety of hemoglobin and myoglobin derivatives has been completed. From the polarized single-crystal absorption spectra we have determined the directions of the electric dipole transition moments, from the natural circular dichroism the magnitudes of the magnetic-dipole transition moments, and from the magnetic circular dichroism the permanent magnetic moments of the excited states. The magnetic circular dichroism has also proven to be an excellent new method of discriminating optical transitions from ground states of different spin multiplicities, thereby permitting a sensitive measure of spin-state populations for reactivity studies. During the past year our main efforts have been directed toward a theoretical analysis of the results with the aim of assigning all of the observed transitions within the framework of the extended Hückel molecular orbital model.

b. Electronic structure of isolated hemes (with Dr. Hanson)

We have begun an investigation of the optical spectra of neat porphyrin and metalloprophyrin crystals in order to provide basic spectroscopic information on the heme chromophore in the

absence of the protein and also as a model system for understanding heme-heme interactions in multiheme proteins. Our effort so far has been concentrated on the metal-free porphyrin, tetraphenylporphin. We find that all of the well-known visible and near ultraviolet transitions are perfectly in-plane polarized. Because of its out-of-plane polarization a near ultraviolet absorption system has been discovered which may be the first observation of an optical transition involving sigma electrons in a free base porphyrin. A more general aspect of this work is that we are exploring the use of phase modulation techniques for the study of anisotropic optical properties of organic crystals.

c. Structure of cobalt-substituted myoglobin (with Drs. Padlan and Yonetani)

Cobalt may be substituted for iron in hemoglobin or myoglobin and yield an oxygen-binding protein which, in the case of hemoglobin, may still bind oxygen cooperatively. In order to investigate the role of the metal in the stereochemical trigger mechanism that is responsible for cooperativity we are investigating the structure of cobalt-substituted sperm-whale myoglobin by X-ray diffraction. Three-dimensional data to 2.8 Å resolution has been collected on crystals of metcoboglobin (Co(III)) and deoxycoboglobin (Co(II)). At the end of the X-ray exposure the deoxy crystals were perfectly intact as judged by their diffraction pattern and polarized optical spectra, while a new optical spectrum was obtained for the exposed met crystals, indicating extensive radiation damage. A difference Fourier synthesis versus acid metmyoglobin has been calculated for the deoxy derivative. Major differences in the two structures include a loss of the iron-bound water molecule, indicating that the metal in deoxycoboglobin is five-coordinate, a loss of the sulfate ion bound to the distal histidine, presumably as a result of the decrease in charge on the metal, and a reorientation of the heme group. Further detailed differences, particularly the movement of the metal position, are now being analyzed.

3. Flavoproteins (with Drs. Ludwig, Hofrichter and Makinen)

We have measured the polarized single-crystal absorption spectra of the oxidized and semiquinone forms of flavodoxin from the bacterium Clostridium MP using a recently-constructed double-beam recording microspectrophotometer. The crystal spectra provide well-defined transition moment directions for the two lowest $\pi \rightarrow \pi^*$ transitions of both the oxidized and semiquinone forms. Because these directions are essentially identical we conclude that

the first two transitions have the same detailed orbital origin in both forms. This result indicates that semiquinone formation takes place by the addition of an electron to the lowest empty pi orbital of the oxidized form with very little electronic rearrangement.

Significance to Biomedical Research and the Programs of the Institute:

A knowledge of the structure of the hemoglobin S fiber that forms in sickled red cells and the kinetics and thermodynamics of its formation should lead to a rational therapy for sickle cell disease. The measurement of melting curves and rate of gelation on microsamples of isolated hemoglobin S provides an important assay for investigating the potential ability of agents to reduce the extent and rate of sickling.

The elucidation of relationships between conformation and electronic structure of the active sites of proteins should lead to a much deeper understanding of how these molecules perform their biological function.

The techniques developed in our investigations may be applied to a wide variety of problems in protein structure and chemistry.

Proposed Course of Project:

We shall continue our study of the structure, kinetics, and thermodynamics of sickling. Future structural experiments will include optical and X-ray diffraction measurements on gels. From further model-building experiments on the computer and the construction of real physical models we will examine in atomic detail potential intermolecular contact sites in the hemoglobin S fiber. In addition to the constraints of the X-ray diffraction, electron microscope, and optical results, we will employ the results of gelation experiments with mutants other than hemoglobin S and chemically-modified hemoglobins in order to construct models of the intermolecular contacts in atomic detail. The definition of these sites could lead to the design of a stereo-specific inhibitor of sickling.

In addition to the present techniques we shall employ light scattering and nuclear magnetic resonance linewidth measurements to study the mechanism of hemoglobin S gelation. We shall also explore other techniques such as natural circular dichroism, densimetry and dilatometry. From simultaneous measurements with this wide variety of techniques we hope to distinguish steps which involve the formation or dissolution of hemoglobin S fibers from steps which involve alignment or disalignment of fibers, and addition or dissolution of monomers to or from aligned fibers.

We shall continue our investigation of the electronic structure of hemes using optical spectroscopy. Our emphasis with the heme protein

spectra will be on measuring low temperature spectra and completing the theoretical analysis, while with the neat porphyrin crystals we shall carry out mainly experimental studies on crystals of iron-porphyrin complexes which bind oxygen reversibly.

Publications:

Hofrichter, J., Hendricker, D. G. and Eaton, W. A.: Structure of hemoglobin S fibers: Optical determination of the molecular orientation in sickled erythrocytes. Proc. Nat. Acad. Sci. USA. 70, 3604-3608, 1973.

Makinen, M. W. and Eaton, W. A.: Optically-detected conformational changes in hemoglobin single crystals. Nature 247, 62-64, 1974.

Stephens, P. J., Sutherland, J. C., Cheng, J. C. and Eaton, W. A.: The study of spin-states of heme proteins by near infrared magnetic circular dichroism. Proc. Int. Conf. Excited States of Biol. Mol., Lisbon, April, 1974. In press.

REPORTED IN PRESS - 1973

Makinen, M. W. and Eaton, W. A.: Polarized single crystal absorption spectra of carboxy- and oxyhemoglobin. Ann. N. Y. Acad. Sci., 206, 210-222, 1973.

Eaton, W. A. and Lovenberg, W.: The iron-sulfur complex in rubredoxin, in Iron-Sulfur Proteins, Vol. II, Molecular Properties, W. Lovenberg, (ed.) (Academic Press, New York, 1973) p. 131-162.

Serial No. NIAMDD-LCP-13
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure and interaction of paramagnetic biomolecules

Previous Serial Number: Same

Principal Investigator: Hideo Kon

Other Investigators: Mitsuo Sato

Cooperating Units: Dr. Daniel W. Nebert, NICHD-LBS
Dr. Albert Szent-Györgyi, Marine Biological Labs, Woods Hole, Massachusetts

Man Years

Total:	1.25
Professional:	1.25
Other:	0

Project Description:

Objectives:

To investigate the electronic structure and the chemical behavior of paramagnetic molecules which occur in biological systems, or which serve as suitable models for natural systems.

Methods Employed:

Electron paramagnetic resonance (epr) absorption was the principal experimental means to investigate the paramagnetic molecules. Extensive use was made of a cryostat covering a wide temperature range including sub-nitrogen temperatures. Nuclear magnetic resonance (nmr) and optical absorption spectroscopy have also been used.

Major Findings:

1. Using the pentacoordinated nitric oxide (NO) complex of Fe(II) meso-tetraphenylporphine (TPP), the mode of interaction of this molecule with the environment (solvent matrix) was studied in detail. It was found that a clear distinction can be made, by the use of epr, be-

tween the two situations, the first in which a specific σ coordination with Fe(II) takes place on the vacant axial site and the second where the solvent molecule interaction with the pentacoordinated complex is more or less non-specific. Based upon this information, it became possible to interpret some of the epr spectra which have been widely observed in the NO derivative of structurally perturbed hemoproteins. For example, the characteristic three line epr pattern observed when some allosteric effector phosphate interacts with NO-hemoglobin can be attributed to the rupture of the bond between the heme and the proximal histidine.

It was also found that the epr spectral pattern of the pentacoordinated NOFe(II)TPP is so sensitive to the difference in the immediate surrounding (especially in the second situation cited above) that epr may be used to probe into the internal motion of NO-iron porphines in various matrices including protein crevices.

2. With Dr. D. W. Nebert

The study of the change in the high spin epr signal (g=8) intensity of the liver microsome P-450 relative to the low spin signal, due to the in vivo treatment with sodium phenobarbital (PB) or 3-methylcolanthren (MC) was extended to rabbit and various strains of rat. In contrast to last year's findings with mouse, subtle strain dependent differences exist in the response to the PB induction in rat and rabbit. In MC induction, the increase was found to associate principally with the high spin state. Also in MC treatment of rat and genetically responsive mouse, the rise in time of the g=8 signal occurred as the amount of the microsome-bound MC started decreasing, suggesting some genetically mediated process involved in the increase in the g=8 signal.

3. With Dr. A. Szent-Györgyi

It had been known that the yellow colored precipitate from a mixture of a p-phenylenediamine and a glyoxal solution (a donor-acceptor couple) dissolves in strong acid with the display of a very short lived deep blue coloration. The direct epr study of the solution containing this transient blue species gave only a remote correlation between the blue material and the paramagnetism, which was expected if the charge transfer produces free radicals. However, by isolating the yellow compound and reacting with HCl gas in dry solid state, it was found that the blue-colored state can be trapped permanently, that there is positively a transition of an electron from the donor to the acceptor under the influence of a strong acid, and that the possibility of O_2 mediated radical formation can be ruled out. This is another example we have found of a donor-acceptor combination, in which the charge transfer originally

associated with an excited electronic state of the complex can be made to be enhanced in degree and be shifted to the ground state by the action of a third molecule.

Significance to Biomedical Research and the Program of the Institute:

These investigations contribute the basic information necessary for understanding the interactions involving metal ions, small organic molecules and the protein in more biologically significant systems.

Proposed Course of Project:

In addition to the investigations being conducted at present, an effort will be made in searching for a model compound for the high spin state (g=8) of cytochrome P-450, which plays an important role in the metabolism of drugs etc., using its characteristic epr signal as a monitor.

Publications:

Kon, H. and Szent-Györgyi, A.: Charge transfer between amine and carbonyl. Proc. Nat. Acad. Sci. USA 70, 3139-3140, 1973.

Nebert, D. W., Robinson, J. R. and Kon, H.: Further studies on genetically mediated differences in monooxygenase activities and spin state of cytochrome P-450 iron from rabbit, rat and mouse liver. J. Biol. Chem. 248, 7637-7647, 1973.

Kon, H.: An interpretation of the three line epr spectrum of nitric oxide hemoproteins and related model systems: The effect of the heme environment. Biochem. In press.

REPORTED IN PRESS - 1973

Nebert, D. W., Considine, N. and Kon, H.: Genetic differences in cytochrome P-450 during induction of mono-oxygenase activities. Drug Metab. & Disp. 1, 231-238, 1973.

Kon, H. and Szent-Györgyi, A.: Interaction of amines and ketone aldehydes and unsaturated aldehydes. Proc. Nat. Acad. Sci. USA 70, 1030-1031, 1973.

Serial No. NIAMDD-LCP-14
1. Chemical Physics
2. Spectroscopy & Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Electronic and molecular structural investigations

Previous Serial Number: Same

Principal Investigator: Ruth McDiarmid

Other Investigators: None

Cooperating Units: None

Man Years

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To describe the electronic and molecular structures of ground and excited states of simple molecules.

Methods Employed:

The measurement and analysis of electronic absorption spectra, predominantly in the ultraviolet. The generation of unstable molecules by pyrolytic techniques. The calculation, with digital computers, of energy levels and geometries to correlate with and further explain the experimental observation.

Major Findings:

The results of these investigations are of significance because of the characteristics of the subject molecules. These molecules are small enough to be theoretically and experimentally tractable yet large enough to embody chemical groups of interest. Most of the work reported involved the ether group.

1. Dimethyl ether is known to manifest several diffuse transitions. Here the 1700 Å transition was shown to arise from a promotion out of the second highest filled molecular orbital. This contrasts with the generally accepted model in which the lower energy transitions of a molecule arise only from promotions out of the highest occupied molecular orbital.

2. The 1900 Å transition of diethyl ether was shown to contain more information than the analogous transition of dimethyl ether. From the small interval vibronic spacing observed in this transition, we were able to demonstrate that the barrier to methyl torsion in this electronic state is greatly reduced from its ground state height.

3. The analysis of the absorption spectra of $(CH_3)_2S$ and $(CD_3)_2S$, the sulfur analog of ether, between 2500 Å and the first ionization potential was completed: Most of the >25 observed bands were arranged in three Rydberg series, the lower energy transitions were demonstrated to be of mixed valence Rydberg character. Two of these lower energy transitions were demonstrated to arise from the second highest filled molecular orbital, analogous to the case in dimethyl ether itself.

4. The spectra of WF_6 and MoF_6 at wavelengths longer than 1100 Å were analyzed. All 7 observed bands of WF_6 and 6 of the 7 bands of MoF_6 were demonstrated to arise from F→Metal 5d t_{2g} promotions. These results can now be compared with our earlier results for ReF_6 to study the effect(s) of electron-electron interaction of these types of electronic transitions.

Significance to Biomedical Research and the Program of the Institute:

This research project seeks to understand the electronic changes a molecule undergoes on absorbing light and, more significantly, the structural alterations that a given electronic change can cause. This study can, if fruitful, provide a basis for a physical interpretation of the relative spectroscopic results obtained in other, more general studies.

Proposed Course of Project:

1. This year we intend to investigate the ultraviolet absorption spectra of several unstable molecules of interest. Initially we hope to generate, observe and analyze $H_2C{=}S$ and HN=NH.

2. There remain several unresolved aspects of the ongoing ethylene investigation, which we hope to resolve.

3. A more formal investigation of the effect of electronic excitation on torsional barriers will be initiated.

Publications:

McDiarmid, R.: On the 1700 Å transition of dimethyl ether. J. Chem. Phys. 60. In press.

McDiarmid, R.: C-O torsional vibrations in the 1900 Å transition of diethyl ether. J. Chem. Phys. In press.

McDiarmid, R.: Assignments of rydberg and valence transitions in the electronic absorption spectrum of dimethyl sulfide. J. Chem. Phys. In press.

ANNUAL REPORT
LABORATORY OF PHYSICAL BIOLOGY
NATIONAL INSTITUTE OF ARTHRITIS, METABOLISM, AND DIGESTIVE DISEASES

The Laboratory of Physical Biology is carrying out studies in a broad range of research areas.

Molecular Structure

Immunoglobulin. Information about the structure of biologically important protein macromolecules can be obtained by high resolution electron microscopy. The size and shape of these molecules can be determined most accurately when they are crystallized into three-dimensional structures. Protein crystals that are large enough can be studied by X-ray diffraction techniques, with such a study leading, under favorable conditions, to a complete three-dimensional placement of the amino acids in the protein molecule. Although the electron microscope can not give comparable detailed information, it can give the rough size and overall shape of the molecule, and its approximate location in the crystal lattice. This information is helpful at the beginning of a detailed X-ray analysis. The electron microscope can also give this type of information for protein crystals too small for X-ray studies, and for crystals that are not sufficiently stable under X-ray bombardment to make a detailed X-ray analysis feasible.

This year, cubic crystals of phosphorylcholine binding Fab' fragments from mouse myeloma immunoglobin protein were studied in the electron microscope. This crystal is unstable under X-ray bombardment so a detailed X-ray analysis was not possible. The space group was found to be $P2_13$ and arrangement of molecules in the unit cell was worked out. (Labaw)

Muscle. To study the structural changes associated with contraction, instrumentation has been developed for recording the spatial distribution of diffracted X-rays electronically. An intense beam of focused X-rays from a rotating anode tube is passed through a muscle preparation and the equatorial diffraction pattern is recorded with a position-sensitive X-ray detector. The patterns obtained from the detector are comparable to those recorded on film; the potential speed of the electronic system is about 100 times faster than film. The method will be used to study the spatial distribution of molecules in resting and active muscle cells, and the influence of active shortening on this distribution. (Yu, St. Onge)

Red cell sickling. Sickle cell anemia is one of the few genetic diseases of man in which the defective molecule has not only been identified (it is a distinctive hemoglobin, HbS), but the abnormal interactions of this molecule can be explained. Work on HbS has advanced on two fronts. With regard to the

chemistry of HbS, it was found that aggregation can be inhibited by inorganic ions according to the classical Hofmeister series and that tetrabutyl ammonium chloride reverses the temperature coefficient of gelation. Another interesting finding is that inhibitors of microtubule formation also prevent HbS microtubules from forming. On the clinical side, more data was collected regarding the effectiveness of oral administration of urea in ameliorating the symptoms of sickle cell crises. These cases were studied in Ghana, West Africa, in collaboration with Dr. Oku Ampofo. (Murayama, Levine)

Cell Membranes

Lipid monolayers and bilayers. A current hypothesis of membrane function is that enzyme mediated transport is critically affected by the "state" of the membrane lipids. To characterize these states, the phase relations of lipid mixtures in monolayers and bilayers have been studied by differential scanning calorimetry. The general rules for predicting the phase relations in complex lipid mixtures in various states of aggregation--anhydrous melts, liquid crystals and aqueous suspensions--have been established, and the influence of cholesterol on the phase transitions of phospholipids has been measured. (Gershfeld)

Mycoplasma membranes. Unlike most microorganisms, the membranes of mycoplasma contain cholesterol. The uptake of cholesterol by isolated mycoplasma membranes requires the presence in the membrane of lipid rather than protein. Although most mycoplasma strains require cholesterol for growth, one (A. laidlawii) does not. An interesting observation in this connection is that the membrane of this strain does not take up extracellular cholesterol when the cell is growing; however, when growth stops, a considerable amount of cholesterol is taken up. (Razin, Gershfeld)

Red cell membrane. A variety of physical techniques indicate that cholesterol modulates the fluidity of lipid bilayers. This idea was followed up by examining the effect of cholesterol on the structure of human erythrocyte membranes. X-ray diffraction was used to assay membrane fluidity. Membranes of normal erythrocytes were compared with those of erythrocytes whose membrane cholesterol had been depleted *in vivo* by the biochemical processes that are involved in the *in vivo* maintenance of normal cholesterol levels. Despite removal of up to 30% of the membrane cholesterol, the hydrocarbon chains of the lipids in the membrane maintain the liquid-like structure found in normal cells down to at least -20°C. This result is consistent with parallel measurements on extracted membrane lipids, which show that at least 35% of the normal cholesterol content must be removed for hydrocarbon chain crystallization to occur at -20°C. Procedures are being developed for the removal of greater percentages of the membrane cholesterol. One of the major difficulties here lies in the unexpected, and possibly important, finding that removal of cholesterol from the membrane is accompanied by the uptake from plasma of cholesterol esters, which are normally completely absent from the membrane. (Gottlieb)

Muscle Contraction

The cross-bridge mechanism. Two new kinetic techniques have been developed for characterizing cross-bridge activity in skeletal muscle cells. In one, the transducers used to record the motion of skinned muscle fiber segments were modified so that the pre-steady, as well as the steady, contracti[illegible] kinetics could be obtained. The pre-steady kinetics indicate that cross bri[illegible]es between reactive sites on the actin and myosin filament are formed quic[illegible] relative to the transit time of the two sites. This recording technique will be used to find out how the cross-bridge mechanism is affected by chemical parameters that modulate the acto-myosin interaction in vitro. (Gulati, Podolsky)

The other new technique is to measure the propagation time of a mechanical impulse down the length of a muscle cell. The magnitude of this time (about 0.1 msec for a 1 cm long fiber) is proportional to the instantaneous number of cross bridges in a muscle cell. This technique will be used to determine how the number of cross bridges changes under various conditions of contraction, which is a critical point in various contraction hypotheses. (Schoenberg)

The activation mechanism. Contraction and relaxation of skeletal muscle cells are controlled by release of calcium from the internal membrane system and by subsequent reaccumulation of this calcium; the mechanical effect of this process depends on the Mg^{2+} level. Measurements of Ca^{45} movement between the internal membranes and the bulk of the muscle cell show that the mechanical effect of Mg^{2+} is due primarily to the acceleration of the calcium reaccumulation process by Mg^{2+} rather than direct effect of Mg^{2+} on the cross-bridge mechanism. (Stephenson)

Morphogenesis

Cellular organelles. Euglena is a cell in which the interaction of enzymes located on a given organelle, as well as the interaction of systems between organelles, can be studied. The organelles can be stratified by centrifugation; the normal spatial distribution is reestablished if the cell is supplied with a minimal growth medium. To analyze this process of active organization, the metabolic pathways of Euglena are being sorted out. The single most abundant compound in the small molecule pool is mannitol; the remainder of the compounds are principally amino acids. The absolute cellular concentration of the different amino acids were established in extracts of Euglena grown on three different carbon sources (glutamic acid, acetate and CO_2 by photosynthesis). The overall distribution was found to be independent of the culture conditions. Those amino acids which are "family heads" (amino acids from which others are synthesized) are predominant. In addition, peptides are found in this fraction. One of these, the most abundant and the most easily separable, has been identified as L-arginyl-L-glutamine. It is present in cells from all three different culture media and has been shown to be an intermediate compound since it is labelled by radioglutamic acid and the

radioactivity is "chased" by non-radioactive carbon sources. (Kempner)

Insect metamorphosis. The transformation of the larval form of an insect to an adult raises the question of whether the enzymes that carry out the same metabolic step in the two forms are the same or different. In the case of the blowfly, muscle aldolase is the same in both forms, but fatbody aldolase is not. Thus the adult insect is clearly more than a simple rearrangement of preexisting larval components. A related problem concerns the fate in the developing adult of the most abundant of the larval soluble proteins, calliphorin. This protein is distinguished by (a) an abundance of aromatic amino acids, and (b) an unusual tertiary structure. At pH 7.5 or higher the hexamer dissociates into monomers, while at pH 6 and below the monomers reassociate; this effect is also concentration and salt dependent. A procedure has been developed for isolation of the protein on a semi-preparative scale, and anti-calliphorin immune serum has also been prepared. (Bauer, Levenbook)

In order to substantiate the hypothesis that larval ribosomes of the blowfly may be carried over intact into the adult fly, uridine triphosphate and free lysine pools have been measured at different stages of the insect life cycle and, on a "per insect" basis, have been found to be remarkably constant during metamorphosis. Experiments are under way to measure the rate of ribosomal RNA synthesis at different developmental stages; these data, when completed, should prove whether or not there is substantial new ribosomal biosynthesis during metamorphosis. (Protzel)

Biological Rhythms

With the possible exception of some subcellular oscillations, all biological rhythms involve at least a basic timing oscillator and a subservient coupled system which is what is monitored and which provides the only handle on the properties of the fundamental repetitive process. Progress has been made in separating oscillator and output contributions in studies of two model controllable rhythms: firefly flashing and human finger-tapping. One interesting finding is that two species of synchronizing fireflies, living in the same area and having almost identical free run periods, have very different pacemaker cycles. Another discovery is that whereas the endogenous oscillator is much less variable than the output in human digital rhythm control, the opposite seems to be true in fireflies. In both firefly and human rhythms, however, peripheral feedback does not appear to be important in period regulation. (E. Buck, L. Mets, J. Buck).

Serial No. NIAMDD-LPB-1
1. Physical Biology
2. Cellular Physics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Mechanism of Muscular Contraction

Previous Serial Number: Same

Principal Investigator: R. J. Podolsky

Other Investigators: Mark Schoenberg
Elizabeth W. Stephenson
Leepo C. Yu
Jagdish Gulati

Cooperating Units: Dr. Jay Wells, Medical Neurology Branch, NINDS
Dr. Richard St. Onge, Department of Physics, University of New Hampshire

Man Years (July 1973 through June 1974)

Total:	7
Professional:	5
Other:	2

Project Description:

Objectives:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.
2. Study of the electrical and chemical parameters involved in the activation of various types of muscle fibers.
3. Measurement of ionic fluxes across the internal membranes of skinned muscle fibers under various conditions.

Major Findings:

1. Mechanical signals move down the length of an activated fiber mainly by an elastic, rather than a viscous, mechanism when one end is displaced at frequencies close to 10^4 cycles/sec. This makes it possible to use the transmission time of such signals to measure the number of actomyosin cross bridges in the fiber.

2. Using this technique, it was found that the stiffness of muscle cells following electrical stimulation does not rise suddenly but follows a slow time course similar to that of force development. Therefore, the rate of cross-bridge formation after stimulation is relatively slow and newly formed cross bridges produce force soon after they are made.

3. In calcium activated skinned muscle fibers, the pre-steady motion at a given relative load is affected by ionic strength. However, the steady contraction speed is insensitive to this parameter, which indicates that cross bridges between reactive sites on the actin and myosin filaments are formed quickly relative to the transit time of the two sites.

4. Rapid depolarization of the internal membrane system of skinned fibers stimulates transient net calcium release from the sarcoplasmic reticulum. Net release depends on the size of the depolarization stimulus and is strongly affected by the calcium reaccumulation rate, which varies with magnesium ion level and with temperature. Release of calcium can be prevented by calcium chelation, which suggests that calcium ion is required for depolarization-induced calcium release.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the molecular mechanism of muscular contraction together with the chemistry of the activation process, can be useful in the rational handling of neuromuscular and cardiovascular disease.

Proposed Course of Project:

1. The influence of various agents (pCa, pH, ionic strength, metal ions, ATP) on the ability of muscle fibers to develop force and to shorten will be examined directly in skinned fiber segments.
2. The influence of these same agents on myofilament spacing and the cross-bridge configuration will be examined in skinned fibers by X-ray diffraction.
3. The effect of motion on the number of cross bridges in intact, electrically-stimulated muscle fibers will be studied by X-ray diffraction.
4. Movement of calcium and magnesium between the myofilament space and the sarcoplasmic reticulum will be studied by tracer methods.

Publications:

Thames, M. D., Teichholz, L. E., and Podolsky, R. J.: Ionic strength and the contraction kinetics of skinned muscle fibers. J. Gen. Physiol. 63:509-530, 1974.

Podolsky, R. J., Gulati, J., and Nolan, A. C.: Contraction transients of skinned muscle fibers. Proc. Nat. Acad. Sci. 71:1516-1519, 1974.

Schoenberg, M., Wells, J. B., and Podolsky, R. J.: The measurement of muscle compliance. J. Gen. Physiol. (In press).

Serial No. NIAMDD-LPB-2
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Sense of Rhythm.

Previous Serial Number: NIAMDD-LPB-2

Principal Investigator: John B. Buck

Other Investigators: Drs. Laurens Mets and Kenneth Friedman, Mr. Charles H. Hanna

Cooperating Units: Mrs. Elisabeth M. Buck, Guest Worker; Dr. Frank A. Hanson, University of Maryland, UMBC, Catonsville, Maryland; Dr. James F. Case, University of California, Santa Barbara, California; Dr. Thomas A. Hopkins, Guest Worker.

Man Years:

Total:	4.5
Professional:	3.5
Other:	1

Project Description:

Objectives:

To understand the mechanism and function of certain rhythmic motor behavior in selected organisms. To understand how oscillatory activity in one organism can be entrained to that in another.

Methods Employed:

Standard electrophysiological and psychophysical. Special computer analyses.

Major Findings:

1. Some success has been achieved in separating the effects of the endogenous pacemaker and motor process during rhythmic voluntary human finger tapping. The variance of the timing cycle is a smooth function of tapping rate and is stable from experiment to experiment in a given individual. In contrast, effector variance fluctuates

widely over relatively short time intervals and is not affected by tapping rate. Right and left hands appear to be controlled by distinct timers, with that of the dominant hand dominating when both hands tap together. (L. Mets and J. Buck).

2. Comparison of flash synchronization mechanisms in two Melanesian firefly species with 1000 msec free run periods has been completed. Each species can be entrained to rhythmic artificial light signals, species A over the period range of 800 to 1800 msec, B only between 900 and 1060 msec. The range of the species B response is set by restriction in possible interflash period duration, though the pacemaker continues to cycle whether or not the animal actually flashes. The pacemaker of species A can be reset in an all-or-none and cycle-by-cycle fashion by external signals. Species B resets only over a much narrower range and then only according to a 1:2 period to phase relation: outside this range, driving results in either reversion to free run flashing or flash inhibition. The two species differ in their pacemakers: In A the flash is triggered at the moment the cycle switches from period-lengthening to period-shortening effects; In B the flash is triggered at a point in the timing cycle 300 msec after that switch has occurred. (J. Buck, E. Buck, F. Hanson, J. Case).

3. In a firefly species in which the female responds to signals delivered in pairs, she responds normally whether the two flashes are delivered exclusively to the same eye or to both eyes serially. She orients toward the second flash of the pair, but is unable to discriminate directionally between signal pairs, as evidenced by the inhibitory effect of a single out-of-phase flash to one eye while the other receives a normal signal pair (J. Case and J. Buck).

4. Cinematographic analysis has shown that a firefly species from highland New Guinea, which emits a high frequency flicker of up to 28 consecutive 60 msec pulses at an average rate of 5/sec, synchronizes by leader-triggering rather than by cycle-by-cycle phase-shifting as in certain lowland species. (K. Friedman).

Significance to NIAMDD Research:

The new data on rhythms and entrainment encourage the hope of eventually defining human pacemaking activity, separating timing from motor steps and understanding the ability to entrain to external pacing.

Proposed Course of Project:

No major changes anticipated.

Publications:

REPORTED IN PRESS - 1972

Halverson, R. C., Case, J. F., Buck, J., and Tiemann, D.: Control of luminescence in Phengodid beetles. J. Insect Physiol. 19: 1327-1339, 1973.

Serial No. NIAMDD-LPB-3
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Dynamic Properties of Cell Membranes and Related Systems

Previous Serial Number: Same

Principal Investigator: N. L. Gershfeld

Other Investigators: K. J. Friedman
K. Tajima
J. N. Weinstein

Cooperating Units: S. Razin, Department of Clinical Microbiology, Hadassah Medical School, Jerusalem, Israel

Man Years (July 1973 through June 1974)

Total:	4.5
Professional:	3.5
Other:	1

Project Description:

Objectives:

(a) To understand the molecular mechanisms underlying membrane assembly and the relation between membrane structure and function.
(b) To establish the physico-chemical basis of cell membrane dynamics

Methods Employed:

General techniques of surface chemistry have been developed to measure equilibrium properties of fluid surfaces and the thermodynamics of very slightly water soluble lipid solutions. Differential scanning calorimetry has been applied to the analysis of phase transitions of phospholipids. A fast-freezing autoradiographic technique is under development for examining structurally heterogeneous lipid monolayer systems.

Major Findings:

(1) The energetics of anhydrous mixtures of cholesterol with lecithins in the liquid, liquid-crystalline and crystalline state indicate that the

more ordered the phospholipid state, the less favored is the cholesterol-phospholipid interaction. Thus, the interaction of cholesterol with anhydrous lecithin becomes less favored in the following order: liquid >> liquid-crystalline >> crystalline.

(2) The lipid composition of the hyphal membrane of the mold Neurospora crassa can be altered via genetic and media manipulations. The role of lipids in determining the electrical properties of these membranes is being studied (Friedman). D. C. resistance measurements as a function of temperature have shown the wild-type N. crassa membrane manifests changes in membrane conductance, in the temperature ranges of 0-8°C and 20-30°C, characteristic of homogeneous phases. However, in the temperature range of 8-20°C there is a dramatically large change in the N. crassa w/t membrane conductance, possibly signifying a phase transition. No concomitant changes in membrane potential were observed.

(3) Conditions have been defined for establishing equilibrium (as contrasted with metastable) states of phospholipid aggregates in water. The energetics of the monolayer to liquid-crystal phase transition for phospholipids has been measured. (Tajima)

(4) Cryogenic and autoradiographic techniques have been developed for determining the rate of lateral diffusion of lipid molecules in condensed monolayers. (Weinstein)

(5) Cholesterol uptake in mycoplasma membranes has been found to depend wholly on the lipid content of the membranes and not membrane protein. Growing cells utilize a mechanism for restricting cholesterol incorporation; this mechanism does not function in non-growing intact cells.

Significance to Biomedical Research and the Program of the Institute:

The role of cholesterol in membrane function is still not understood. Our studies on the cholesterol-lipid mixtures should provide a rational basis for the separation of the physical from the biochemical effects of cholesterol.

Proposed Course of Project:

(1) Neurospora lipid mutants will be used to test whether membrane lipid composition influences the D.C. conductance.
(2) Stable equilibrium suspensions of lecithin-cholesterol mixtures in water will be used to test the influence these lipid surfaces have on the activity of various membrane-associated enzyme systems.
(3) Diffusion studies of lipid molecules in homogeneous and heterogeneous lipid films will be continued.
(4) The mechanism of cholesterol rejection by mycoplasma membranes will continue to be studied.

Publications:

Pagano, R. E. and Gershfeld, N. L.: Phase separation in cholester -dipalmitoyl lecithin mixed films and the condensing effect. J. Coll Interface Sci. 44: 382-383, 1973.

Gershfeld, N. L., Wormser, M., and Razin, S.: Cholesterol in mycoplasma membranes. I. Kinetics and equilibrium studies of cholesterol uptake by the cell membrane of Acholeplasma laidlawii. Biochim. Biophys. Acta (In press).

Razin, S., Wormser, M., and Gershfeld, N. L.: Cholesterol in mycoplasma membranes. II. Components of A. laidlawii cell membranes responsible for cholesterol binding. Biochim. Biophys. Acta (In press).

REPORTED IN PRESS - 1973

Razin, S., Rottem, S., Hasin, M., and Gershfeld, N. L.: Binding of exogenous proteins and lipids to mycoplasma membranes. Ann. N. Y. Acad. Sci. 225: 28-37, 1973.

Gershfeld, N. L.: Thermodynamics and experimental methods for equilibrium studies with lipid monolayers. In Korn, E. D. (ed.): Methods in Membrane Biology. New York, Plenum Publishing Corp, 1974, pp. 69-104.

Serial No. NIAMDD-LPB-4
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Physical Chemistry

Previous Serial Number: Same

Principal Investigator: M. H. Gottlieb

Other Investigators: None

Cooperating Units: Dr. E. D. Eanes, LBS/NIDR
Dr. H. Yeh, LC/NIAMDD

Man Years (July 1973 through June 1974)

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To determine the role played by cholesterol in certain properties of cell membranes.

Methods Employed:

X-ray diffraction, nuclear magnetic resonance, calorimetry

Major Findings:

The overall approach is to deplete erythrocytes of cholesterol *in vitro* and to compare the properties of the depleted cells with those of normal cells. The literature describes two types of treatment of erythrocytes which remove cholesterol. The first, involving incubation with dilute aqueous dispersions of the phospholipid lecithin, was rejected on finding that lipids other than cholesterol are removed from the membrane, and that lecithin from the dispersion was taken up by the membrane. The second procedure utilizes the physiological mechanisms responsible for the maintenance of normal *in vivo* erythrocyte cholesterol levels, i.e. the exchange of cholesterol between the cell membrane and the serum lipoproteins. The serum is first preincubated at 37°C, during which time a

large fraction of the lipoprotein cholesterol becomes esterified. Subsequent incubation of erythrocytes with this cholesterol-depleted serum results in the removal of a large portion of the erythrocyte membrane cholesterol.

A study of this second procedure revealed that while up to about 25% of the membrane cholesterol is removed without otherwise modifying the membrane, further cholesterol removal is accompanied by the uptake from the serum of cholesteryl esters (which are normally completely absent from the membrane).

X-ray diffraction patterns of lipids extracted from (normal) erythrocyte membranes show that the hydrocarbon chains have a liquid-like flexibility at temperatures down to at least -20°C; removal of about 1/2 of the cholesterol from the extracted lipids results in crystallization of some of the hydrocarbon chains at -20°C, and removal of all of the cholesterol leads to hydrocarbon chain crystallization at +20°C.

X-ray diffraction patterns from whole erythrocyte membranes indicate that the hydrocarbon chains have a liquid-like flexibility down to at least -20°C, both in normal membranes and those depleted of up to 1/3 of their cholesterol. The NMR spectra of membranes depleted of 1/3 their cholesterol were completely similar to those of cells of normal cholesterol content.

These data are consistent with other indications that one function of cholesterol in cell membranes is to maintain the liquidity of the membrane.

Proposed Course of the Project:

The uptake of cholesteryl esters by erythrocyte membranes during incubation with plasma will be studied in detail, both to develop procedures for depleting cells of larger portions of their cholesterol without introducing cholesteryl esters, and as a possibly important process per se.

Significance to Biomedical Research and the Program of the Institute:

Studies on the effects of cholesterol contents of erythrocyte membranes on their properties should contribute to the understanding of hemolytic anemias, as well as to general membrane structure. The understanding of the mechanism of the uptake of cholesteryl esters from serum by erythrocytes may shed light on the reasons for the normal absence of such compounds from erythrocyte membranes. It may also have a significance for the process of cholesteryl ester deposition on arterial walls, a process associated with atherosclerosis.

Publications:

Gottlieb, M. H. and Eanes, E. D.: Coexistence of liquid crystalline and rigid crystalline phases in lecithin-water mixtures. Biophys. J. 14: (May), 1974.

Gottlieb, M. H.: Binding of alkaline and alkaline earth cations in lecithin mesophases. J. Colloid Interface Sci. (In press).

Serial No. NIAMDD-LPB-5
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of metabolic activity in microorganisms

Previous Serial Number: NIAMDD-LPB-5

Principal Investigator: Ellis S. Kempner

Other Investigators: Jay H. Miller and Talaat E. Shehata

Cooperating Units: None

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives:

An understanding of the control of metabolic activity in unicellular organisms.

Methods Employed:

Culture of microorganisms in the presence of radioactive or other compounds; chemical and physical fractionation of the cellular materials by ultracentrifugation, chromatography and chemical extraction. Microscopic examination of cells by time-lapse video micrography.

Major Findings:

Studies of the recovery of stratified Euglena cells described last vear have been continued. The loss of cells from culture during the first five hours post-centrifugation was prevented specifically by molybdate ion. The protection from loss is independent of both sodium ion and exposure of Euglena to molybdate prior to centrifugation. Neither the glass culture flask or the presence of phosphate (both of which could have chemical interactions with molybdate) are involved in the loss of centrifuged cells.

Classical studies of cell volume and macromolecular composition in bacteria during shifts between rich and minimal growth media have re-

vealed interesting relationships between macromolecular synthesis and cell division. In Euglena, previous studies have rigorously established both cell volume and chemical composition of cells grown in a minimal culture medium. Present work has already shown that Euglena grown in a rich medium have a considerably larger cell size. It is not due to increased osmotic strength since this decreases mean cell volume. Further analysis of Euglena during changes in growth medium will allow a comparison of these eucaryotic organisms with the procaryotic bacteria.

The composition of the amino acid pool of Euglena gracilis has been studied in cultures with three different carbon sources. The general distribution of amino acids and the dipeptide arginylglutamine show only minor changes. There is therefore a strong homeostatic control of these metabolic intermediates.

Significance to NIAMDD Research:

This work is aiding the development of a system for studying differentiated cellular structure in Euglena gracilis with the technical ease usual with bacteria. Using this system, new insight into the structure and function of cells is being gained.

Proposed Course of Project:

To study the metabolic relationships of synthetic processes in Euglena to the highly organized cellular structure which has already been elucidated.

Publications:

Kempner, E. S. and Miller, J. H.: The molecular biology of Euglena gracilis. IX. Amino acid pool composition. J. Protozool. In press.

REPORTED IN PRESS - 1973

Kempner, E. S. and Miller, J. H.: The molecular biology of Euglena gracilis. VIII. Glycitols and monosaccharides. J. Protozool. 19: 678-681, 1972.

Serial No. NIAMDD-LPB-6
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Investigation of the macromolecular organization of living matter.

Previous Serial Number: Same

Principal Investigator: L. W. Labaw

Other Investigators: None

Cooperating Units: None

Man Years (July 1973 through June 1974)

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives:

To gain information about the macromolecules that are essential constituents of living matter and to see how they are arranged in the structures they form. To study certain of these macromolecules, such as viruses and other proteins, in purified form after isolation from the living material. To improve the resolution of the electron microscope and to interpret the way in which images are formed near its limit of resolution.

Methods Employed:

Electron microscopy of microorganisms, cells and tissues in suspension or thinly sectioned. Physicochemical characterization of macromolecular components isolated from such material using electron microscope, X-ray diffraction, and similar established techniques.

Major Findings:

Cubic crystals of phosphorylcholine-binding Fab' fragments from mouse myeloma (McPC603) immunoglobulin protein have been studied in the electron microscope. Preliminary X-ray diffraction studies showed that these

crystals belong to either the P23 or $P2_13$ space group, but their radiation sensitivity precluded the more detailed X-ray analysis necessary to distinguish between the two possible space groups. The EM work showed the correct space group to be $P2_13$, and placed the twelve molecules in the unit cell. They are arranged in four groups of three, such that the three molecules in each of these four groups have three-fold rotational symmetry around a cube body diagonal in the same way that three molecules are positioned around the three-fold rotation axes in the hexagonal Fab' crystal studied previously. Assuming a partial specific volume of 0.74, the Fab' molecules occupy only 22% of the cubic crystal volume.

Significance to Biomedical Research and the Program of the Institute:

The use of the electron microscope to determine the crystal structure of protein crystals is particularly important when applied to crystals which cannot be grown large enough for a more complete structure study by X-rays or which are too sensitive to X-ray degradation.

With increased emphasis on fine structure in electron microscopy, it is pertinent to examine the mechanism of image formation to define the conditions under which one can be assured that observed periodicities on images in the electron microscope have a direct spatial relationship with the object being examined when one is operating near the resolution limit of the instrument.

Proposed Course of Project:

To investigate other protein crystals in the electron microscope using replica, negative staining, and direct transmission techniques to determine their structures.

Publications:

None

Serial No. NIAMDD-LPB-7
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical Changes during Development of Blowflies

Previous Serial Number: NIAMDD-LPB-7

Principal Investigator: L. Levenbook

Other Investigators: A. Bauer, S. Sridhara and A. Protzel

Cooperating Units: Dr. P. S. Chen (University of Zurich, Switzerland)
Dr. H. Fales NHLI-LC-8

Man Years:

Total:	2.75
Professional:	2.75
Other:	0

Project Description:

Objectives:

1. An understanding of the interrelationship(s) between ribosomes of a polymorphic developing system as illustrated in blowfly metamorphosis.

2. An elucidation of the structure and function of the insect protein calliphorin.

3. Identification of paragonial factor from male Drosophila melanogaster.

Methods Employed:

Routine physical methods for the isolation of ribosomes, precursor pools, and calliphorin. Routine chemical procedures for the study of ribosomes, purified proteins, and organic chemistry of carbohydrates on a micro scale.

Major Findings:

1. (a) Earlier evidence for the degradation of fatbody rRNA into a lower molecular weight insoluble form has been confirmed by isotopic experiments that also suggest elimination of the degraded RNA at adult emergence. (b) Ribosomal precursor pool sizes have been

measured and found to be surprisingly constant during blowfly adult development. (c) The isotopic data suggest that extracellular and intracellular ribosomes of developing adult blowflies derive from the same ribosomal population, and that to a significant degree larval ribosomes maintain their integrity during metamorphosis.

2. The exact structure of the hormone-like paragonial material of D. melanogaster is proving unexpectedly difficult to elucidate. There is now good evidence that the compound is galactopyranosyl-1-glycerol coupled at an undetermined position to phosphoethanolamine.

3. Calliphorin, the major storage protein of many insects, is composed of six dissimilar subunits which readily undergo association and disassociation. This phenomenon is only partially understood and/or controllable. The hexamer molecular weight, by gel electrophoresis and ultracentrifuge is close to 0.5 X 10^6 daltons, and the isolation point is about 5.2. Antibody to purified calliphorin has been prepared, and employed for checking purity.

Significance to NIAMDD Research:

The developing adult insect exhibits drastic tissue breakdown and extensive histogenesis; it thus serves as a model system for the study of ubiquitous cellular phenomena which may be obscure in man, but can be discerned in the insect by virtue of their occurring in an exaggerated form. This is well illustrated by the finding of blowfly extra-cellular ribosomes; if these were to occur at the site of (say) trauma in vertebrates, they would be difficult to find because of probable removal by the blood stream.

Proposed Course of Project:

Continuation of studies on developmental biochemistry of ribosomes, the structure and biological function of calliphorin, and the structure of paragonial substance.

Publications:

Levenbook, L., Bauer, A. C., and Shigematsu, H.: Fructose diphosphate aldolase during growth and development of the blowfly Phormia regina (Meigen). III. Physicochemical comparison of the larval and adult muscle enzymes. Arch. Biochem. Biophys. 157: 625-631, 1973.

Sridhara, S. and Levenbook, L: Extracellular ribosomes during metamorphosis in the blowfly Calliphoran erythrocephala. Biochem. Biophys. Res. Comm. 53: 1253-1259, 1973.

Sridhara, S. and Levenbook, L.: The contribution of the fatbody to RNA and ribosomal changes during development of the blowfly Calliphora erythrocephala (Meig.). Devel. Biol. In press.

Serial No. NIAMDD-LPB-8
1. Physical Biology
2. Comparative Physiology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Molecular Mechanism of Human Red Cell Sickling and Unsickling *in vitro* and *in vivo*.

Previous Serial No.: NIAMDD-LPB-8

Principal Investigator: Makio Murayama

Other Investigators: Dr. Alan S. Levine

Cooperating Units: Dr. Oku Ampofo, Tetteh Quarsh Memorial Hospital, Ministry of Health, Mampong-Akwapim, Ghana, West Africa

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Objectives:

1. To find a molecular mechanism of "unsickling" of erythrocytes from sickle cell anemia patients.

2. To evaluate thermodynamic constants: the enthalpy change in the thermal (endothermic) aggregation of sickle cell hemoglobin (Hb S); the entropy change in the thermal aggregation of Hb S; and the volume of activation in the endothermic aggregation of Hb S.

3. To investigate further the effects of the constituents of the Hofmeister Series on the blockage and reversal of sickling using S hemolysate and S erythrocytes.

4. To study the effects of inhibitors of microtubule formation because:

   a. Hb S forms microtubule upon sickling.
   b. There is evidence for hydrophobic interactions being responsible for Hb S microtubule formation.
   c. Microtubules in general are formed by hydrophobic interactions.

Methods Employed:

Gelation measurements of Hb S; Equilibrium solubility measurements of Hb S; Nephelometric method for the determination of rate of aggregation of Hb S; Differential interference (Nomarski) optics microscopy and time lapse cinephotomicrography of sickling and unsickling processes.

Major Findings:

Hb S aggregation can be inhibited by inorganic ions according to the classical Hofmeister series ($Cl^- < NO_3^- < Br^- << I^- << SCN^-$). Tetrabutyl ammonium chloride has been found to reverse the temperature effect on gelation (never before observed). This phenomenon is due to the hydrophobic properties of the tetrabutyl-ammonium ion which, at low temperature, causes highly structured water, making it thermodynamically favorable for Hb S to aggregate. However, at 37°, the tetrabutyl-ammonium ion breaks water structure and preferentially participates in hydrophobic interaction with Hb S, thereby sterically hindering Hb S molecules from interacting with each other. The entropy term, which contributes most to the free energy of Hb S polymerization, dictates that it is energetically unfavorable for aggregation. Preliminary findings show that inhibitors of microtubule formation can also prevent Hb S microtubules from forming.

Significance to NIAMDD Research:

Sickle cell anemia is a hereditary, hemolytic anemia, hence significant to the NIAMDD mission. My theory has been applied at the bed side with success here as well as in Africa (oral urea therapy and oral urea prophylaxis), Sao Paulo, Brazil and Havana, Cuba. When urea has been used improperly, as in NIH-NHLI study, this could lead to failure.

Proposed Course of Project:

The extract of Fagara xantheroides will be investigated further. Initial isolation of the active ingredient(s) and then identification and the usual process of working out the structure and so forth. Then knowing the structure that might cause desickling, we might figure out the process of sickling.

Publications:

Murayama, M.: Thermal (or endothermic) aggregation of sickle cell hemoglobin (Hb S) during sickling. Thrombosis Diathesis Haemorrhagica Supplement 53: 45-52, 1973.

Levine, A. S., Hasegawa, F., and Murayama, M.: The influence of solutes and solvent structure on aggregation of deoxy-sickle cell hemoglobin. J. Mol. Med. In press.

Murayama, M.: Molecular aspects of tactoid formation in man and animals. Ann. N. Y. Acad. Sci. In press.

REPORTED IN PRESS - 1973

Murayama, M.: My individual science. Thrombosis Diathesis Haemorrhagica Supplement 53: 37-44, 1973.

ANNUAL REPORT SUMMARY
LABORATORY OF BIOPHYSICAL CHEMISTRY

The main objective of this laboratory has been, as in previous years, the study of the correlation of structure of protein molecules to their functions. The proteins studied were the oxygen-carrying proteins, those participating in muscular contraction, and blood clot formation. To varying extent, the primary, secondary and tertiary structure of these proteins came into the field of our inquiries as the following summary of the work reported illustrates.

HEMOGLOBIN: The oxidation of hemoglobin from the ferrous to the ferric (met) state in vitro is ordinarily accomplished by the use of potassium ferricyanide sodium nitrate or oxygen in acid solution. A reagent first reported in 1912 is hydroxylamine and very few publications have appeared on its use. We are now studying the oxidation of hemoglobin with hydroxylamine. We find that one hydroxylamine oxidizes two iron atoms. The rate of oxidation by hydroxylamine is fast enough for most uses and much slower than that of oxidation by ferricyanide. Since the product of the reaction is NH_3, this neglected reaction of hydroxylamine appears to be ideally suited for the oxidation of hemoglobin. (Simpson, Saroff)

Minton extended the thermodynamic model for aggregation ("gelation") of sickle-cell hemoglobin to treat aggregation in binary mixtures of the four hemoglobin variants S,A (normal), C_{Harlem}, and Korle-Bu and the aggregation in solutions of HbS in the presence of an additive. A new and unique instrument has been constructed for automatic measurement of the minimum gelling concentration (MGC), an important parameter characterizing the process of aggregation in HbS solutions. The instrument is designed to detect the sharp increase in viscosity which occurs upon increasing the concentration of HbS above its MGC. This instrument is expected to give experimental data with precision and rapidity on the effects of temperature, oxygen saturation, and various additives upon the aggregation of sickle-cell hemoglobin. (Minton).

MUSCLE PROTEINS: The ability of muscle to switch activity off when not in use, and on again when contraction is required, involves the interaction of two contractile and four regulatory proteins. Detailed understanding of this interaction could not only clarify the normal operation but also suggest areas of potential malfunction in disease and possible sites of therapeutic approach to such problems as spasticity or weakness.

New techniques of labelling purified muscle proteins with ^{125}I, utilizing soluble and solid-state lactoperoxidase, to allow studies of the binding of ^{125}I-labelled proteins to F-actin have been developed by Kominz and co-workers. A direct correlation has been found between the binding of ^{125}I-labelled tropomyosin to F-actin, and the inhibition of acto-heavy meromyosin ATPase activity. When tropomyosin neither binds to F-actin nor inhibits the acto-heavy meromyosin ATPase, addition of troponin-I restores both the binding and the inhibition. Since troponin-I binds to F-actin but not to tropomyosin, this result suggests that troponin-I modifies F-actin in some way which strengthens the tropomyosin binding. Troponin-C reverses

the troponin-I effects to a greater extent in the presence of Ca^{++} than in the absence of Ca^{++}. (Eaton, Kominz, Tsukui and Eisenberg)

CLOTTING PROTEINS: This laboratory has been concerned for years with the protein-protein interaction involved in blood coagulation. The research is oriented particularly to the mechanisms of the thrombin conversion of fibrinogen to fibrin and the ensuing enzymatic cross-linking of fibrin monomers to form the actual physiological material constituting a "blood clot". Since blood coagulation is responsible for "coronaries" and "strokes", an understanding of these reactions could lead towards eventual control of these diseases.

Lamprey fibrinogen appeared to be a good material for the study of cross-bonding of fibrinogen. Lamprey thrombin releases two peptides from this fibrinogen while bovine thrombin only releases one. This circumstance offers an opportunity to determine if the release of the peptides has an effect on the subsequent cross-linking. (Gladner, Murtaugh)

Lamprey fibrinogen appears structurally very different from any fibrinogen reported to date. Although it possesses 3 types of chains, α, β and γ (2 of each), as in all fibrinogens, the α-chain is more than twice the molecular weight of the γ-chain. A detailed study is now in progress to characterize lamprey fibrinogen. (Gladner, Murtaugh, in cooperation with M. Lewis)

One of our main objectives this year concerned clot retraction which is considered an essential process in wound healing. It is generally assumed that the contractile proteins of platelets, (myosin and actin), are responsible for clot retraction. Our starting point was that myosin, or actin, or both, should bind to fibrin if these proteins are involved to shrink the clot. Our studies revealed that F-actin binds to fibrin in a 2 to 1 molar proportion but not to fibrinogen. During the course of these studies, we found that thrombin hydrolyzed actin. To our surprise, we found that thrombin, known to be specific for Arg-Gly bonds, did not split actin's two Arg-Gly bonds -- instead split Arg-Ala Arg-His and Arg-Lys bonds.

There are two interesting aspects of these findings. Since ADP-actin was found to be cleaved faster at the Lys-Ala and Arg-His bonds than ATP-actin, we surmise that the terminal phosphate of ATP is bound to these Arg and Lys residues, thereby slowing down the action of thrombin. This is the first time that the actual residues can be identified which bind ATP.

The fact that actin does not bind to fibrinogen, only to fibrin, has an ominous implication. It is now known that transglutaminase (activated Factor XIII) can clot fibrinogen directly without thrombin first generating fibrin molecules. The implication of our finding is that fibrinogen clot may not undergo clot retraction and thereby the first step in wound healing is impaired. (Laki, Muszbek, Gladner)

A NEW AMINO ACID: Irreverre and co-workers discovered a new amino acid identified and characterized as 3-(2-furoyl)-L-alanine from the hydrolyzed extracts of seeds from Koelreuteria paniculata commonly known as golden rain tree. It is the first time that an amino acid with a furan ring was found. It is of interest to note that 4 laboratories have discovered the same amino acid from different sources in about the same time: R. L. M. Synge's group in England; A. Ichihara's group in Japan; and P. Larson in Denmark.

The precursor of furoylalanine in this plant is probably ascorbalamic acid or furoylalanine tied to ascorbic acid (vitamin C) since the precursor gives both aspartic acid and furoylalanine on acid treatment. Furoylalanine and its precursor, ascorbalamic acid (furoylalanine + ascorbic acid) is present in some foods from plants; therefore, the elucidation of their biochemical degradation and metabolism in the body is important. (Irreverre)

Serial No. NIAMDD LBC-1
1. Biophysical Chemistry
2. Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanisms of muscular and cellular contraction.

Previous Serial No.: NIAMDD LBC-1

Principal Investigator: Dr. David R. Kominz

Other Investigators: Dr. Ryoko Tsukui, Visiting Associate, Univ. of Tokyo (through July, 1973)
Dr. Barbra Eaton (starting Sept. 17, 1973)
Mr. Henry Wolff

Outside Collaborators: Dr. Evan Eisenberg, LB NHLI, Dr. Warren Evans, LB, NCI, Mr. John Powell, CSL, DCRT, Dr. Ned Feder, LEP, NIAMDD, Dr. Fred Fay, Univ. of Mass. Medical School, Worcester, Mass.

Man Years
Total: 3.5
Professional: 2.0
Other: 1.5

Project Description:

Objectives: To advance the understanding of muscular and cellular contractility.

Methods: Experiments aim at correlating enzymatic activity with physical properties of purified contractile and regulatory proteins, of suspensions of various living cells, and of glycerinated muscle fibers. New techniques of labelling purified muscle proteins with ^{125}I, utilizing soluble and solid-state lactoperoxidase, have been employed to allow studies of the binding of ^{125}I-labelled proteins to F-actin. Rapid tape storage of cell-sizing data has made possible kinetic curves of cell contraction at two minute time intervals. An interface has been constructed to connect the Intertechnique multichannel analyzer and teletype via an acoustic coupler to the NIH PDP-10 computer for rapid analysis of the data (Powell). Solutions and suspensions are also studied in a combined spectrophotometer and pH stat, and glycerinated fibers are monitored for length and tension while mounted for laser or other optical examination.

Major Findings and Proposed Course of Studies: 1. There is a direct correlation between the binding of ^{125}I-labelled tropomyosin to F-actin, and the inhibition of acto-heavy meromyosin ATPase activity. Under conditions

where tropomyosin neither binds to F-actin nor inhibits the acto-heavy meromyosin ATPase, addition of troponin-I restores both the binding and the inhibition. Since troponin-I binds to F-actin but not to tropomyosin, this result suggests that troponin-I modifies F-actin in some way which strengthens the tropomyosin binding. Troponin-C reverses the troponin-I effects to a greater extent in the presence of Ca^{++} than in the absence of Ca^{++}. (Eaton, Kominz, Tsukui and Eisenberg).

2. Under conditions where tropomyosin alone binds poorly to F-actin, a direct correlation exists between the binding of heavy meromyosin and the binding of ^{125}I-labelled tropomyosin to F-actin. This suggests that instead of preventing tropomyosin binding to F-actin, rigor bonds between heavy meromyosin and F-actin can enhance it. (Kominz).

3. Mature guinea pig polymorphonuclear neutrophils contract in response to acetyl choline or adrenaline. The contractile responses follow different kinetics in the two cases. Immature cells from guinea pig bone marrow or from human acute myeloid leukemia do not contract in the presence of these agents. (Kominz, Wolff and Evans).

4. From commercial nylon threads whose individual filaments are of uniform diameter in the range of 8-20μ, microscopic rods of uniform length could be prepared with a Sorvall tissue chopper. Preparations of different lengths served as standards to test the ability of cell-sizing equipment to measure volume and length distributions, and to construct standard curves to use with living and fixed cells. (Kominz, Feder and Fay).

Projects Proposed: 1. Using combined binding and ATPase studies, we will examine further the interactions between the troponin components, tropomyosin, actin and heavy meromyosin.

2. The kinetics of various cells' contractile response to pharmacological reagents will be studied. In collaboration with Dr. Evans, the role of cellular maturity will be explored.

3. To the instrumentation developed by Dr. Goldstein for monitoring sarcomere length and tension on glycerinated fibers, the added capability of measuring ATPase activity will be pursued.

Significance to Biomedical Research and the Programs of the Institute: The ability to switch muscle activity off when not in use, and on again when contraction is required, involves the interaction of two contractile and four regulatory components. Detailed understanding of this interaction could not only clarify the normal operation but also suggest areas of potential malfunction in disease and possible sites of therapeutic approach to such problems as spasticity or weakness.

A basis for hormonal control of the inflammatory reaction has recently been discovered: the release of lysosomal enzymes from polymorphonuclear

neutrophils has been found to be accelerated by acetylcholine and cyclic GMP, and inhibited by catecholamines and cyclic AMP. The loss of volume found by us with acetylcholine could be ascribed to release of lysosomal contents, but the smaller, more rapid volume decrease with adrenaline is not readily explained; perhaps a fundamental response to hormonal binding is involved. Additionally it would be of great interest to pinpoint precisely the nature of the process transferring apparently mature but non-interacting bone marrow neutrophiles into responsive cells in peritoneal cavity exudates.

Honors and Awards: None

Publications:

Kominz, D. R. and Gröschel-Steward, U.: Antibody-dependent size changes of myofibrils and isolated smooth muscle cells. J. Mechanochem. Cell Motility 2: 181-191, 1973.

Serial No. NIAMDD-LBC-2
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical studies on physiologically important micro and macromolecules in living systems.

Previous Serial No.: NIAMDD-LBC-5.

Principal Investigator: Filadelfo Irreverre

Technical Assistance: Mr. Edwin Wilson

Cooperating Units: H. M. Fales, LC, NHLI
T. Sun, LC, NHLI
E. Sokoloski, LC, NHLI

Man Years
Total: 2
Professional: 2

Project Description:

Objectives: Man, animals and plants are interrelated to a certain extent and are made up of rigid molecular structures and circulating fluids carrying a myriad of substances, both macro and micromolecules. For a number of years, studies have been directed on (1) the nature of some of the unknown nitrogenous molecules, and (2) the relationships of these compounds to biochemical reactions in the living systems.

Methods: Modern chemical and biochemical techniques have been routinely employed, such as TLC, paper and column chromatography, automated amino acid analysis, gel separation and filtration, paper and gel electrophoresis, UV, infrared and mass spectrometry, NMR, ultracentrifugation, radioisotopic techniques and enzymatic analysis. When necessary, development of new procedures of isolation, detection or micro-assay has been undertaken.

Major Findings: We have discovered a new amino acid which we have identified and characterized as 3-(2-furoyl)-L-alanine from the hydrolyzed extracts of seeds from Koelreuteria paniculata commonly known as golden rain tree. It is the first time that an amino acid with a furan ring was found. It is of interest to note that 4 laboratories have discovered the same amino acid from different sources in such a short time: (1) R. L. M. Synge's group in England (Phytochemistry 12, 707, 1973); (2) A. Ichihara's group in Japan (Tetrahedron Letters 1, 37, 1973); (3) our work in the USA, and (4) P. Olesen Larson in Denmark (personal communication). We further found that

furoylalanine occurs free in the young seeds (green) of Koelreuteria paniculata but in the mature seeds, (black), it could only be detected after acid treatment. The precursor of furoylalanine in this plant is probably ascorbalamic acid or furoylalanine tied to ascorbic acid (vitamin C) (Phytochemistry 12, 707, 1973), since isolation of a small amount of the precursor gives both aspartic acid and furoylalanine on acid treatment.

Developed a quantitative determination of furoylalanine by an automated column chromatography which separates it from other amino acids. We are now applying this procedure to biological materials such as urine and blood since the precursor of furoylalanine is present in foods (plant source).

Significance to bio-medical research and the program of the Institute: Since furoylalanine and its precursor, ascorbalamic acid (furoylalanine + ascorbic acid), is present in some foods from plants, the elucidation of their biochemical degradation and metabolism in the body will contribute to our knowledge of nutrition and biochemistry.

Proposed course of project: Biochemical studies of physiologically interesting compounds in living systems will continue. Special emphasis will be directed to biochemical and enzymatic studies of these unusual and interesting compounds.

Honors and awards: None.

Publications:

1. Tabor, H., Tabor, C. W. and Irreverre, F.: Quantitative determination of aliphatic diamines and polyamines by an automated liquid chromatography procedure. Anal. Biochem. 55: 457-467, 1973.

Serial No. NIAMDD LBC-3
1. Biophysical Chemistry
2. Bioenergetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The action of thrombin on fibrinogen and the physiological mechanism of action of the peptides released during blood clotting.

Previous Serial No.: NIAMDD LBC-7

Principal Investigator: A. J. Osbahr

Man Years:
Total: 1.0
Professional: 1.0

Project Description:

Objectives: To understand the structural basis for the enzymatic action of thrombin on fibrinogen, and for the physiological action of the peptides released from fibrinogen during blood clotting; to gain insights into the possible role of these peptides in hemostasis, vasoconstriction and pulmonary hypertension.

Methods: The methods applied to study these systems were those of structure determination and analysis and techniques to evaluate homogeneity and purity of the protein and peptide systems under investigation. High voltage electrophoresis, liquid column chromatography, spectrophotometric anallysis, ultracentrifugation and regular and SDS gel electrophoresis are some of the methods applied to the analysis of the protein interactions.

Proteolytic enzymes were employed for "active site" determinations. Physiological monitoring was performed in the rat by means of intracarotid cannulation or microscopic visualization of bleeding arterioles of scarified tail.

Major Findings and Proposed Course of Studies: Earlier investigations "in vitro" on rat uterus indicated that the fibrinopeptides potentiate bradykinin induced contraction of rat uterus muscle; degradation of canine peptide A produced an amino acid fragment which contained the biological activity.

"In vivo" studies on the bovine fibrinopeptide B demonstrate that it possesses the ability to produce hemostasis in rat tail arterioles. The peptide produces a 10-fold decrease in the time of bleeding at a 10^{-4} M concentration. The hemostasis occurs immediately and incubation with the peptide was unnecessary. These findings suggest that hemostasis is due to vasoconstriction since the coagulation time remains constant as the bleeding time

decreases with increasing concentration of peptide. The vasoconstriction would thus aid the hemostatic process by enabling the arterioles to narrow at the injury site, compressing and anchoring the clot plug and facilitating the ultimate termination of bleeding.

In other "in vivo" experiments, canine fibrinopeptide A was demonstrated to be an active vasopressor substance. The effect of intravenous injection of the peptide was a unique rhythmic vasoconstriction, up to 30 min. in duration. A dose of 2.5×10^{-2} μ moles of peptide produced an increase in mean blood pressure of 19.0 mm of mercury. When split in half by chymotrypsin, the N-terminal portion of the peptide retained the biological activity and the C-terminal half was inactive.

Since a possible mechanism for fibrinopeptide vasoconstriction might be direct action on the contractile proteins, its effect on the superprecipitation of myosin B was investigated. With 10^{-4} M bovine peptide B, the rate of superprecipitation was increased 12-fold while the ATPase activity doubled. To allow this direct action to occur, the negatively charged peptide must pass through the membrane barrier by a process like pinocytosis. Otherwise the peptide must act by attachment to some smooth muscle receptor or indirectly via another regulatory system.

Significance of Biomedical Research and the Program of the Institute:

Our studies indicate a relationship between the peptides from the blood clotting protein fibrinogen and physiological processes in blood circulation. It is hoped that investigation of these physiologically active peptides may result in a deeper understanding of the mechanisms associated with hemostasis, vasoconstriction and blood coagulation.

The more we know about the physiological activity of the peptides, the easier could be the task of sorting out some lesser known mechanisms involved in related diseases, such as the etiology of respiratory distress syndrome and certain diseases associated with high blood pressure. The presence of peptides from fibrinogen due to intravascular clotting might underly many systemic manifestations associated with these diseases. We hope to demonstrate the presence of fibrinopeptides as a possible agent for such vasoconstriction.

Proposed Course of Project:

The action of thrombin on modified fibrinogens will be evaluated. The physiological role of the native and modified peptides from fibrinogen will be further investigated.

The effect of chemical modification of acid residues as well as other functional groups in the biologically active peptides will be explored.

Honors and Awards: None

Publications:

Osbahr, A. J. and Colman, R. W.: Structure-activity relationships of the peptides released from canine fibrinogen by thrombin. Biochim et Biophys Acta 336: 273-282, 1974.

Serial No. NIAMDD-LBC-4
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Proteins, enzymes and peptides involved in blood coagulation. Proteases of pituitary glands and their possible relationship to pituitary hormones.

Previous Serial No.: NIAMDD-LBC-4

Principal Investigator: Jules A. Gladner

Other Investigators: Mrs. Patricia A. Murtaugh

Cooperating Units: Dr. John Halver, Chief, Fish Nutrition Lab., Fish and Wildlife Service, Dept. of Interior, Cook, Washington; Dr. Marc S. Lewis, NEI, NIH; Dr. Peter Condliffe, NIAMDD-LNE; Dr. L. Kohn, NIAMDD-LBP.

Man Years:
Total: 4
Professional: 4

Project Description:

Objectives: The major investigation involves the protein-protein interaction concerned with blood coagulation. This is oriented particularly to the molecular biological mechanisms of the thrombin conversion of fibrinogen to fibrin plus the ensuing enzymatic cross-linking of fibrin monomers to form the actual physiological material constituting a "blood clot". In addition, we have begun investigating the possible enzymatic reversal of this cross-linking reaction.

Another possible type mechanistic study has begun involving an investigation of the proteases of the pituitary gland. Past reports in the literature have reported large molecular weight pituitary hormones. Our object is to ascertain if there exist pro-pituitary hormones and if pituitary proteases, of the limited protease variety, play a role in converting pituitary pro-hormones to an active or more active type.

Methods: Methods employed consisted of protein-enzyme fractionation, purification, characterization and modification. Where needed, methods of physical biochemistry and analytical chemistry were utilized, such as but not limited to, SDS gel electrophoresis and automatic amino acid analysis. Physiological techniques were utilized to examine the biological activity of various peptides. As applicable, new techniques were developed.

Major Findings: Thrombin conversion of fibrinogen to fibrin and cross-linking of fibrin monomers. We have continued focusing our attention to these reactions, especially cross-linking, to the lower species. In addition to our investigations of Columbia River salmon reported last year, we can now add our discoveries obtained utilizing the plasma of the lamprey eel, the first vertebrate.

Previously, investigators have shown that lamprey fibrinogen can be converted to fibrin via thrombin. If its own thrombin (lamprey) is used, 2 peptides are released, A and B. However, if a higher species of thrombin is used, (bovine), then only 1 peptide is released, peptide B, which is one of the largest reported to date, containing 40 amino acid residues and a molecular weight of about 5000. One of the interesting aspects of studying the cross-linking of lamprey fibrin formed via bovine thrombin was the opportunity, for the first time, to determine if release of peptide exercised any effect on the subsequent cross-linking reactions as to rate, lysis times, etc. The opportunity for this type of study has never presented itself before.

Lamprey fibrinogen, from a biomolecular standpoint, appears structurally very different from any fibrinogen reported to date. Although it possesses 3 types of chains, α, β and γ (2 of each), as in all fibrinogens, the α-chain is more than twice the molecular weight of the γ-chain. A detailed study (with Dr. Marc Lewis, see below) is now in progress to characterize biophysically lamprey fibrinogen.

Our cross-linking studies with Factor XIII of lamprey fibrins (i.e., produced either by bovine or lamprey thrombin) give the following results: (1) Lamprey plasma contains Factor XIII, a transglutaminase enzyme which cross-links its fibrin in a similar manner to that of the higher species. However, certain rate differences are apparent. The γ-chains cross-link to γ-γ dimers within seconds while the α-chains cross-link much more slowly forming α-polymers at a much slower rate. As a matter of fact, additional Factor XIII is required to completely cross-link all the α-chains. This could be attributed to the fact that while α-chains normally cross-link at a slower rate than the γ-chains, (in all species), the extremely slow rate in lamprey α- cross-linking is retarded further sterically due to the larger size of the chain. (2) The lysis times (i.e., strength of the cross-linked fibrin) appears to be a function of γ-γ cross-links, in contrast to α-cross-linking reported for higher species. (Murtaugh, Gladner, Halver).

Physico-chemical experiments are in progress to characterize the lamprey fibrinogen via ultracentrifugal analysis. Assuming a molecular weight of approximately 350,000, S_{20}=8, for most fibrinogens, one can examine the following hypothetical molecular structure for lamprey fibrinogen. Three postulates exist: (1) The α-chains are actually larger than any heretofore shown. Therefore, the molecular weight will be larger by about 100 to 150,000 than any fibrinogens previously reported. (2) The α-chains are of normal size but already contain intra-cross-links between γ-glutamyl-ϵ-amino lysyl residues (i.e., a Factor XIII type link); thus, upon reduction of all the molecule's disulfite bonds, one would obtain a α-α dimer. This dimer

would account for the high molecular weight, ca 117,000. As we have shown, it can cross-link further under proper conditions. The molecular weight of the fibrinogen would be the same as in all other species. Further proof of this hypothesis would be the isolation from this fibrinogen of a peptide(s) containing the γ-glutamyl-ε-amino lysyl links; methods for this isolation exist. (3) Not very probable, but the possibility of a fibrinogen dimer formed by intermolecular cross-linking between 2 α-chains, one from ea order of 700-800,000 and a much higher $S_{20,w}$. (Gladner, Lewis).

Enzymatic reversal of fibrin cross-linking. An enzyme "procollagen peptidase" is responsible for conversion of procollagen to collagen and experiments with synthetic substrates indicate the possible rupture of a γ-glutamyl-ε-amino lysyl bond during this conversion. We have utilized completely cross-linked bovine fibrin as substrate to reaction with this enzyme. Preliminary evidence exists that there is some hydrolysis of the α-polymer back to single α-chains utilizing disc gel electrophoresis for analysis. The enzyme is still impure (protease contaminated) and further experiments await an enzyme of higher purity. (Gladner, Kohn).

Modified fibrinogens, fibrins and other proteins as substrates for thrombin. As indicated in last year's report, the above and other proteins are being modified by succinylation. To this, we have now added the proteins actin and egg white lysozyme which contain 2 Arg-Gly bonds. These are the bonds hydrolyzed by thrombin in fibrinogen. The change to more negative regions may influence thrombin action on these bonds. (Gladner, Lewis).

Proteases of the pituitary. Initial studies of bovine pituitary extracts and homogenates show the distinct presence of proteolytic enzymes not susceptible to DFP. The enzymes are probably catheptic in nature and further investigations are proceeding. (Gladner, Condliffe).

Significance to biomedical research and the program of the Institute: By the utilization of blood systems of lower species, one might observe reactions which are masked or even only slightly residual in man, but a malfunction of same could lead to undefined reasons for plasma component(s) maladies. Any information concerning thrombin action is most vital to eventual regulation of the action of this enzyme.

Proposed course of project: We anticipate proceeding along the lines outlined in the above report with emphasis placed on major results as they occur.

Honors and awards: None.

Publications:

1. Murtaugh, P. A., Halver, J. E., and Gladner, J. A.: Cross-linking of salmon fibrinogen and fibrin by Factor XIII and transglutaminase. Biochem. Biophys. Res. Commun. 54: 849-855, 1973.

Serial No. NIAMDD LBC-5
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Intermolecular interactions in physical biochemistry.

Previous Serial Number: LBC-2

Principal Investigator: Allen P. Minton

Man Years:
Total: 1.0
Professional: 1.0

Project Description:

Objectives:

1. To increase our knowledge about and understanding of the process by which sickle-cell hemoglobin aggregates; and to use this knowledge and understanding to develop chemical means for inhibiting the aggregation of sickle-cell hemoglobin *in vivo*, thereby alleviating symptoms of sickle-cell disease.

2. To ascertain the structural basis of cooperative ligand binding in hemoglobin.

Methods:

Theoretical studies employ methods of classical and statistical thermodynamics together with computer techniques for fitting theoretical model functions to experimental data. Experimental studies of the dependence of hemoglobin aggregation upon experimental variables utilize a viscosity-based assay described below.

Major Findings and Proposed Course of Studies:

The thermodynamic model for aggregation ("gelation") of sickle-cell hemoglobin described in last year's project report (NIAMDD LBC-2) was limited to homogeneous solutions of deoxygenated sickle-cell hemoglobin (Hb S). The model has been extended to treat two additional experimentally studied cases:

a) Aggregation in binary mixtures of the four hemoglobin variants S, A (normal), C_{Harlem}, and Korle-Bu. Analysis of published data has provided not only direct information about contributions to the free energy of aggregation due to amino acid substitutions at the key sites $\beta 6$ and β_{73}, but also indirect information about the nature of intermolecular contacts and molecular

orientation in the aggregate.

b) Aggregation in solutions of HbS in the presence of an additive. The extended model permits a molecular interpretation of published data on the effects of various additives upon aggregation and a molecular definition of the clinically important concepts of efficiency and specificity of drug action.

In collaboration with Ira Tice of the BEIB instrument fabrication facility, a prototype has been constructed of a new and unique instrument for automatic measurement of the minimum gelling concentration (MGC), an important parameter characterizing the process of aggregation in Hb S solutions. The instrument is designed to detect the sharp increase in viscosity which occurs upon increasing the concentration of Hb S above its MGC.

During the past year we have demonstrated (partly in collaboration with K. Imai of Osaka University) that only one of the two kinds of simple models commonly used to describe cooperative ligand binding in hemoglobin is consistent with selected experimental data in the literature, thus resolving a long-standing controversy over the relative utility of the two models.

In collaboration with H. A. Saroff, a completely general method for the evaluation of the free energy of interaction in cooperative ligand binding and its dependence upon ligand saturation has been developed.

During the coming year our studies will focus on the following areas:
1) Acquisition of experimental data on the effects of temperature, oxygen saturation, and various additives upon the aggregation of sickle-cell hemoglobin.

2) Extension of the thermodynamic model for aggregation to include solutions of partially oxygenated sickle-cell hemoglobin solutions.

3) Further studies of cooperative ligand binding in hemoglobin, with emphasis upon the effect of partial oxidation of hemes and the role of Bohr sites.

Significance to biomedical research and the programs of the Institute: The extensions of the thermodynamic model for aggregation of sickle-cell hemoglobin constitute a significant advance in our understanding of the aggregation process. In addition, the analysis of additive effects provides important guidelines for clinical investigators in the selection of candidate substances for clinical trial as *in vivo* inhibitors of Hb S aggregation.

It is anticipated that the new instrument for automatic measurement of the MGC will provide an assay of unprecedented precision and rapidity for assessment of the aggregation-inhibiting properties of various additives.

The studies on cooperative oxygen binding in hemoglobin dispose of

some common misconceptions in the field and point the way to a proper understanding of structure-function relationships in hemoglobin.

Honors and Awards: None.

Publications:

1. Minton, Allen P.: A thermodynamic model for gelation of sickle-cell hemoglobin. J. Mol. Biol. 82: 483-498, 1974.

2. Minton, Allen P.: Oxygen binding in cyanmet hybrid and normal hemoglobins: applicability of sequential and two-state concerted models. Science 184: 577-579, 1974.

3. Minton, Allen P. and Imai, K.: The three-state model: a minimal allosteric description of homotropic and heterotropic effects in the binding of ligands to hemoglobin. Proc. Natl. Acad. Sci. U.S. 71: 1418-1421, 1974.

4. Minton, Allen P.: Solubility relationships in binary mixtures of hemoglobin variants. Application to the "gelation" of sickle-cell hemoglobin. In press Biophys. Chem.

Serial No. NIAMDD LBC-6
1. Biophysical Chemistry
2. Macromolecules
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The elucidation of the structure and interactions of biologically important macromolecules.

Previous Serial Number: LBC-3

Principal Investigator: H. A. Saroff

Other Investigators: Dr. R. B. Simpson and H. L. Wolff (1/2 year)

Cooperating Units: Dr. J. Preer, Federal City College, Washington, D.C.; Clinical Endocrinology Branch, NIAMDD

Man Years:
Total: 3
Professional: 2 1/2
Other: 1/2

Project Description:

Objectives: The main objective of this laboratory is an elucidation of the structure of naturally occurring macromolecules based on the methods of physical chemistry. Macromolecules are studied primarily in solution and the emphasis is on the accumulation of data on the state of the molecule with respect to environment in solution. Gross features of structures as well as fine detail, may be observed in the study of macromolecules in solution.

Methods: At present, we are concerned with methods of observing macromolecules in solution. In our studies aimed at finer details of protein structure, the methods used involve the measurements of the binding of small molecules or ions to specific sites on the protein molecule, nuclear magnetic resonance (on model compounds, so far), optical rotatory dispersion measurements and other spectral techniques. For other properties of the protein molecules, we employ the ultracentrifugal, light scattering, electrophoretic and diffusion techniques.

Major Findings and Proposed Course of Studies: The role of 2,3 Diphosphoglyceric acid (DPG) in the action of hemoglobin is complicated by its own binding properties with other ions in solution as well as those with the hemoglobin tetramer. We are attempting to rationalize our data as well as the large amount of published data in this area. One elusive point in this picture is the species of DPG which binds to the hemoglobin tetramer. The DPG (5-) anion appears most reasonable at present and allows the construction of a

unified model for the action of hemoglobin. (Saroff)

A general formulation for the energetics of the cooperative oxygenation of hemoglobin has been presented. The consequences of this formulation suggest some interesting limitations on the energetics of cooperative systems in biological processes. (Minton, Saroff)

The oxidation of ferrous to ferric (met) hemoglobin in vitro is ordinarily accomplished by the use of potassium ferricyanide, sodium nitrate or oxygen in acid solution. A reagent first reported in 1912 is hydroxylamine and very few publications have appeared on its use. We are now studying the oxidation of hemoglobin with hydroxylamine. We find that one hydroxylamine oxidizes two iron atoms. The rate of oxidation by hydroxylamine is fast enough for most uses and much slower than that of oxidation by ferricyanide. Since the product of the reaction is NH_3 this neglected reaction of hydroxylamine appears to be ideally suited for the oxidation of hemoglobin. (Simpson, Saroff)

Studies on aqueous solutions of fatty acids in aqueous solution were recorded to show that the dimerization constant of fatty acids in water is very small. (Simpson)

Studies are being continued on the binding of ions to clusters of ionizable groups in model compounds. (Preer, Saroff)

We have presented models to rationalize some of the binding data collected by the Clinical Endocrinology Branch. (Robbins, Edelhoch, Saroff)

Significance to Biomedical Research and the Programs of the Institute:

Our emphasis is on the relationship between chemical properties and the structure of macromolecules. Once these properties are properly related, the attack on the relationship between structure and function can proceed on a solid basis. The proteins and macromolecules studied are either enzymes or those of interest in biological systems. Our systems are highly controlled with reference to the conditions of the protein environment and the bio-medical significance of our findings derive from extrapolation to the biological system.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-LBC-7
1. Biophysical Chemistry
2. Physical Biochemistry
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: A study of clot retraction.

Previous Serial No.: NIAMDD-LBC-6

Principal Investigators: K. Laki
L. Muszbek, Visiting Associate, Hungary

Technical Assistance: Clyde R. Miller, Jr., Summer Aide

Man Years
Total: 2
Professional: 1-5/6

Project Description:

Objectives: Our objective in the current project is to begin a systematic study of the process of clot retraction. This is the last stage in the process of blood coagulation and the first step in wound healing. According to current views, the actin and myosin-like proteins of the disintegrating platelets are involved. Clot retraction then imitates muscular contraction.

Methods: Methods employed involve enzyme, protein isolation, purification, the tools of protein chemistry such as ultracentrifugation, gel electrophoresis, and amino acid analysis. Enzymatic degradations, radioisotopic techniques, thin layer chromatography and others are also utilized.

Major Findings: When fibrinogen is clotted by thrombin in the presence of F-actin, the clot incorporates the actin filaments. This is evident from electron microscopic pictures and from gel electrophoresis. The specificity of the actin-fibrin interaction is further demonstrated by the finding that the mixed clot dissolves in urea, reforms when the urea is dialyzed out. During these studies, we found that in the absence of Ca-ions and at pH 8.0, thrombin fragmented actin to two smaller peptides and to two larger pieces (MW 26,000 and 10,000) by splitting 3 peptide bonds between Arg(28)-Ala(29), Arg(29)-His(40) and Lys(113)-Ala(114). This was a surprising finding because based on the known specificity, thrombin was expected to split between the 2 Arg-Gly sequences in actin. This finding will help to reevaluate the specificity of thrombin.

Two other interesting aspects of this work are emerging: (1) From the observation that F-actin was digested faster than G-actin while nucleotide-

free actins were digested with equal speeds, we surmise that the terminal phosphate of ATP is bound to Arg(39) and Lys(113). This is the first time that we have an inkling of what residues are involved in binding the terminal phosphate of ATP and how a portion of the actin chain folds.

(2) The other aspects of this work concern the fact that F-actin does not bind to fibrinogen. As we reported years ago, transglutaminase can directly clot fibrinogen. This process is now recognized as an alternate, pathological pathway to clot formation. Our experiments suggest that such a clot will not undergo clot retraction because actin filaments do not bind to it. In other words, the first step in would healing would not come into operation.

Significance to bio-medical research and the program of the Institute: Clot retraction is a vital, last step in blood coagulation. If it turns out that indeed actin and myosin-like proteins are involved in this process, then clarification of clot retraction will lead to a better understanding of clot retraction in health and disease, and muscular contraction, as well.

Proposed course of project: We are proposing to study how actin interacts with clots formed by transglutaminase and whether the large split products of actin are still capable of polymerization and to interact with myosin.

Honors and awards: The James F. Mitchell Foundation Award for Heart and Vascular Research, November 16, 1973; Member of the Board American-Hungarian Cultural Center; Council Member of the American Heart Association.

Publications:

1. Laki, K. and Alving, R. E.: Sulfhydryl groups and the ATPase activity of actin. J. Mechanochem. Cell Motility 2: 45-49, 1973.

2. Laki, K.: Fibrinogen and metastases. J. Med. 5: 32-37, 1974.

3. Laki, K.: Actin as an energy transducer. J. Theor. Biol. 44: 117-130, 1974.

4. Muszbek, L. and Laki, K.: The cleavage of actin by thrombin. Proc. Natl. Acad. Sci. U.S., In press.

ANNUAL REPORT SUMMARY
Laboratory of Molecular Biology

The work of the Laboratory of Molecular Biology is directed to an explanation of biological processes at the molecular level and to an understanding of the physical and chemical foundations of biological processes. Areas of investigation in the laboratory include study of the mechanisms of replication of DNA and of genetic recombination, studies of enzymes involved in recombination, characterization of mammalian and bacterial viruses by genetic analysis, studies of the molecular basis of viral properties, studies of the mechanism of protein synthesis, physical and chemical studies of the structure and properties of polynucleotides and nucleic acids, synthetic and spectroscopic investigation of polynucleotides, characterization of protein-DNA interaction in chromatin, investigation of protein structure by X-ray diffraction, and theoretical studies of nerve membrane characteristics. Major advances in several of these areas have been made by members of the Laboratory during the past year.

Transcription of chromatin. It has been shown for the first time that chromatin isolated from duck reticulocytes can serve as a template for the production of globin RNA in vitro when transcribed with RNA polymerase from E. coli. Chromatin isolated from tissues not producing globin message in vivo do not serve as a template for its production in vitro. Globin message is detected by hybridization to a globin-specific radioactive DNA probe which is complementary to the globin message sequence. These observations have been extended to a quantitative comparison of the template activity of chromatin from duck reticulocytes and erythrocytes. The former produces globin RNA to the extent of about one part in 10^4 when transcribed in vitro. The latter is almost completely inactive as a template, as expected for chromatin isolated from a tissue not actively producing globin message.

It has previously been shown that about half the DNA of chromatin is accessible to a variety of chemical probes, including the enzyme staphylococcal nuclease, and the polycation polylysine. Use has been made of this reactivity to separate chromatin into two distinct classes of double stranded regions, "covered DNA" and "open DNA". Covered reticulocyte DNA anneals with about 20% more of a globin DNA probe than does open DNA. The difference arises because a portion of the globin probe sequence is absent from open regions. It is concluded that a portion of the actively transcribed globin gene is not contained in the open region, and is therefore covered by protein. In contrast, the open and covered DNA regions isolated from erythrocyte chromatin show no such difference, suggesting that the presence of the protein covered region is connected in some way with active transcription of globin sequences.

Chromatin contains the information for tissue-specific transcription. It is possible to show that the information for specific reassembly is also present. If reticulocyte chromatin is dialyzed into solvents containing high concentration of salt and urea, the proteins are dissociated from the DNA and may be isolated. If these proteins are now added to a purified preparation of duck DNA, and the mixture dialyzed back into low salt, the reconstituted chromatin is a template for globin message production with an efficiency close to that of the starting material. (Felsenfeld, Axel, Melchior, Jr., Stannard, Sollner-Webb)

In related studies a DNA ligase has been purified ca. 7000-fold from rat liver. The ligase seals single-strand nicks in DNA with any of the four usual bases on either the 3' - or 5'-sides. It requires ATP and a divalent cation (Mg^{++} or Mn^{++}) for activity. Poly (G) and poly (I) inhibit ligase activity; several other polyribonucleotides are not inhibitory. The molecular weight of the ligase is estimated from gel filtration to be ca. 10×10^4 daltons. The activity of the ligase on polylysine complexes with DNA has been examined to aid in interpreting experiments planned to test ligase activity on chromatin. The ligase has the same maximal velocity on uncovered regions of DNA-polylysine complexes as it does on DNA. It has little or no activity on "nicks" covered by polylysine. The "polynucleotide kinase" from rat liver nuclei has been purified about 400-fold relative to liver extracts. (Zimmerman, Boswell, McKinley).

Studies on DNA replication

1) Replication of E1 plasmid DNA in vitro

A soluble system in which closed-circular DNA molecules of colicin E1 plasmid initiate and complete a round of semiconservative replication of DNA has been developed. Cell extracts were prepared from E. coli carrying colicin E1 plasmid. The DNA in extracts is almost exclusively closed-circular Col E1 DNA. Labeled deoxyribonucleotides are incorporated into DNA in extracts. Col E1 DNA is the sole DNA product, and is composed of completely replicated molecules and a class of replicative intermediates. The intermediates carry an average of approximately two pieces of DNA fragments which are approximately 6S and are not covalently attached to the parental DNA strands. The replication is initiated on closed-circular molecules and the complete molecules are synthesized semi-conservatively. The DNA synthesis depends on the four ribonucleoside triphosphates and is sensitive to rifampicin. A round of DNA replication, once initiated, is completed in the presence of rifampicin indicating that RNA synthesis is involved in the initiation of Col E1 DNA replication. Most of the replicated molecules are isolated in super-coiled structures. These results indicate that this soluble system is capable of carrying out the entire processes of Col E1 DNA replication.

The addition of 10% glycerol and/or 2 mM spermidine to cell extracts blocks synthesis of completely replicated molecules while enhancing synthesis of molecules containing newly synthesized 6S DNA fragments. These molecules are early replicative intermediates. Their synthesis is sensitive to rifampicin and depends on RNA synthesis. RNA components are linked to the 6S DNA molecules.

The structures of molecules of colicin E1 plasmid DNA that are in the process of being replicated in vitro were examined electron microscopically. Circular molecules containing a loop of 7% of the length of the molecules were the major class of replicating molecules. The location of the loop was determined by treating with restriction endonuclease EcoR1 which introduced one unique double-strand break in the colicin E1 plasmid DNA molecules. The loops had a specific location with two branch points at approximately 17 and 24% of the molecular length from the endonuclease sensitive site. Molecules

with a larger loop were observed with a preparation labeled with 5-bromodeoxyuridine and enriched for these molecules. One of the branch points in these molecules was located at approximately 17% from the endonuclease sensitive site independent of the size of the loops. These results indicate that the origin of replication of the plasmid DNA is located within the small loop and the replication proceeds unidirectionally. The molecules that had completed a round of replication had the monomeric twisted circular structure. (Tomizawa, Sakakibara, Sugino, Satterthwait)

2) Replication of ϕX174 and E. coli DNA in vitro

The conversion of ϕX174 single-stranded DNA to duplex DNA is catalyzed by crude extracts of E. coli in a reaction dependent on the products of the dnaB, C(D), E (DNA polymerase III) and G genes and not dependent on the product of the dnaA gene, DNA polymerase I, DNA polymerase II, or RNA polymerase. The involvement of these gene products in this reaction was shown by the increased thermolability of ϕX174 DNA-dependent DNA synthesis in extracts from dna temperature sensitive cells when compared to extracts from temperature resistant revertant cells. Thus the stimulation of inactive crude extracts by fractions from wild-type cells provided complementation assays for the purification of the dna B, C(D), E and G gene products. The identity of proteins isolated by these assays has been established by the isolation of dna B, C(D), E and G gene products from dna B, C, E, and G temperature sensitive cells, respectively, and the demonstration in each case that the temperature sensitive gene product was more thermolabile than the wild-type product. The dna B, C(D) and G gene products have been partially characterized. DNA-dependent and independent ribonucleoside triphosphatase activities are part of or closely associated with the dna B gene product. To date no other enzymatic activities can be attributed to these proteins.

The isolation of all of the proteins required for this reaction is in progress. The mixture of purified dna B, C(D), E amd G gene products alone does not catalyze detectable DNA synthesis in the presence of ϕX174 DNA. Fractionation of the crude extract yields three protein fractions each of which is required in addition to purified dna B C(D), E and G gene products for ϕX174 DNA-dependent deoxynucleotide incorporation. Using this defined in vitro DNA replicating system, the properties of these proteins, their interactions with each other, nucleoside triphosphates, and DNA are being studied to determine their roles in DNA replication. This work is being done in collaboration with J. Hurwitz. (Wicker)

3) DNA ligase mutants of E.coli and DNA synthesis.

Extracts of two lig mutants contain no assayable ligase at 42°. Paradoxically, both mutants are able to carry out UV repair and recombination, processes presumed to require DNA ligase, and both are able to covalently circularize linear λ phage DNA in vivo at 42°. Both of these lig mutants accumulate Okazaki fragments, believed to be intermediates in discontinuous DNA replication, but only one of these lig mutants fails to grow at 42°. By genetically introducing a lop mutation which increases by a factor of five the number, but not type, of molecules of defective DNA ligase in the lig mutants, it has been shown that both mutants produce small amounts of ligase

at 42°. i.e., they are not absolutely defective. The small increase in defective ligase in the inviable mutant is sufficient to allow growth at 42°. Thus, the paradoxical behavior of these mutants can still be accounted for by postulating that lig is the only locus for DNA ligase in E. coli, but that the processes of replication, repair and recombination of DNA in this organism require differing amounts of DNA ligase. (Gottesman, Gellert)

DNA Recombination

1) Studies on "reverse" mutants of phage λ.

The recombination proficient λ phage variant known as "reverse" was derived from a λ phage deleted for recombination functions. It has been shown that this phage carries a substitution of DNA recombination genes derived from the E. coli chromosome. Studies in collaboration with A. J. Clark indicate that this phage carries the gene or genes coding for the RecE recombination pathway in E. coli, including the gene which codes for the newly discovered Exonuclease VIII. Work in progress indicates that the genes for this recombination pathway are derived from a previously undetected defective prophage. (Gottesman, Mizuuchi)

2) In vitro studies on the site-specific recombination system (Int) of phage λ.

An E. coli mutant which gives rise to λ lysogens at reduced frequency following infection has been isolated. This strain is capable of supporting Int-mediated recombination at a normal frequency in phage by phage crosses, suggesting that the strain has an altered attachment site for λ. The further characterization of this strain and a search for other mutant strains, perhaps lacking a host function necessary for integration, is in progress.

It has previously been shown that λ Int protein (integrase) can be radiochemically purified from λ-infected E. coli (Mizuuchi, unpublished), but no biological activity has yet been demonstrated for this protein in vitro. Development of an assay which will detect in vitro Int-promoted recombinants is in progress. (Mizuuchi)

Functional Interaction of Bacterial Viruses with their Hosts.

1) The isolation and characterization of P1 prophages which carry bacterial genes and which are deleted for P1 genes.

Among variants of P1 carrying bacterial pro (proline-biosynthesis) genes, one type has been intensively studied. This P1-pro prophage exists as a circular plasmid consisting of (a) a full complement of P1 genes starting with the gene 2 region, (b) a duplication of the gene 2 region and (c) the bacterial pro genes. Thus, a gene 2 region occurs at both junctions of phage and bacterial DNA. When the prophage is induced, its DNA is replicated and packaged. The entire structure is too large to be packaged within a single P1 phage. However, the encapsidation of P1 DNA including both gene 2 regions can occur, resulting in a normal P1 (pro$^-$) phage. The reciprocal

event, the encapsidation of the bacterial pro genes, bordered on both sides by gene 2 regions, results in the production of pro$^+$ transducing particles.

The pro$^+$ transductants produced by infection with the latter type particle carry the gene 2 region but no other P1 genes. Preliminary mapping experiments indicate that the pro$^+$ genes in these transductants are located on the E. coli chromosome near the his region (quite distant from the normal pro location). The possibility that the gene 2 region may be responsible for promoting recombinations between P1 and the host chromosome is being investigated. (Rosner)

2) The structure of λCM.

The structure of λCM, a bacteriophage λ variant which carries a determinant for resistance to chloramphenicol, has been elucidated. Heteroduplex mapping shows that the CM determinant is inserted into the λ chromosome within the b2 region. The insertion is about 5% the size of λ or about 1.7 million daltons.

The CM determinant is lost from λCM when grown vegetatively at a frequency of about 2% per λ per cycle of growth. However, the loss is not complete; portions of the original insertion remain as observed both by heteroduplex mapping and CsCl equilibrium centrifugation. The recombinational mechanism responsible for this loss of CM is being investigated. (Rosner, Gottesman)

Oncovirology

1) Genetic Analysis of SV40.

Seventy six temperature sensitive mutants of SV40 have been isolated. A new complementation technique was developed which is applicable to all animal viruses. The SV40 mutants can be divided into four complementation groups, A, B, C, and D and a fifth group, BC, which fails to complement with both B and C mutants.

The mutants can be divided into "early mutants", those which fail to synthesize viral DNA at the restrictive temperature, classes A and D, and "late mutants", those which can synthesize viral DNA, classes B, C, and BC. It has been shown that A function is required continuously for viral DNA synthesis to proceed at the restrictive temperature, i.e., preincubation at the permissive temperature followed by a shift to the restrictive temperature results in shut off of viral DNA synthesis. Furthermore, the shut off has been shown to result from the inability to start new rounds of viral DNA replication since replicative intermediate forms do continue to replicate to form mature DNA molecules.

The block in D mutants appears to precede the establishment of viral DNA synthesis. Incubation at the permissive temperature, followed by shift to restrictive conditions does not shut off subsequent viral DNA synthesis provided the incubation has proceeded at the permissive temperature for at least 10 to 20 hours. Yet at 20 hours at the permissive temperature viral DNA synthesis has not yet begun.

Transformation studies using Chinese hamster cells have been initiated. Preliminary results suggest that only A function is required for either the establishment or maintenance of the transformed state. The implication of this finding is that a biochemical understanding of the A function will lead to a biochemical understanding of the oncogenic process. (Martin, Chou, Saral, Avila, Brown).

2) Early stages of Murine Leukemia Virus Infection.

Purified ^{3}H-uridine labelled Rauscher leukemia virus, SLV9, was infected with mouse embryo fibroblasts or normal rat kidney cells. At various times of infection, cells were separated into nuclear and cytoplasmic fractions by treatment with sodium deoxycholate-Tween-40 and nucleic acid extracted by SDS-phenol-chloroform treatment. All radioactivity recovered was acid insoluble and alkaline sensitive. Between 45 ot 70 min after infection, a transient and synchronized shift of the radioactive peak toward the RNA-DNA hybrid region occurred in both the nuclear and the cytoplasmic fractions. The density of the cytoplasmic hybrid shifted to 1.56 (RNA-about 50%) while the sedimentation rate decreased by 36S to 14S. However, the density of the nuclear hybrid shifted from 1.58 to 1.48 (RNA=57 to 17% respectively) while its sedimentation rate remained at about 61S. Both of the hybrids in the nuclear and the cytoplasmic fractions still showed hybrid density after heat denaturation. A model is proposed here: In the early stages of infection, the viral RNA is translocated into the nucleus, with conservation of the whole 70S viral RNA genome in an RNA-DNA hybrid molecule. In the nucleus, DNA synthesis proceeds on these hybrids leading to the formation of a double stranded "viral" DNA and allowing the integration of the viral information. Alternatively, the RNA-DNA hybrid could be integrated directly. (Takano)

Energy Conversion in Biology, Especially Muscle Contraction.

A detailed formalism has been developed that is required for the proper formulation and application of any model of the "sliding filament" type. Topics that have been worked out include steady and transient mechanics, work produced, ATP flux, thermodynamic efficiency, multiple actin sites, the near-equilibrium special case, stochastics, noise, and various special cases. It is planned in the next year to compare the predictions of particular models with experiment, and to extend the formalism to active transport. (Hill, Chen, Ben-Naim)

Calorimetry of Biological Systems

1) Application of calorimetry to cells growing in tissue culture.

A differential microcalorimeter designed to accept the sterile, disposable plastic Petri dishes (35 x 10 mm) used in tissue culture has been constructed. A well thermostatted environment confers long term thermal stability on the instrument so that observations may be carried out over a period of several days and that changes in the rate of heat production of 1.5 microwatts occurring over a five hour period may be detected. The differential scanning calorimeter described last year has been extensively calibrated

and tested. Differences in the thermal output during growth of 3T3 cells and 3T3 cells transformed with SV40 virus have been found. The magnitude of these thermal effects are proportional to cell number and para-synchrony in thermal output has been observed. (Ross)

2) Thermodynamics of Hemoglobin S Gelation.

Deoxyhemoglobin S in concentrated solutions aggregates and forms an ordered, birefringent phase, the so-called gel. It is this gel which distorts red cells upon deoxygenation, forming bizarre and sickled shapes. This system exhibits a temperature-induced phase transition which is being studied by a wide variety of physical techniques.

The enthalpy change for the transition has been determined by differential scanning calorimetry and the data obtained suggest the presence of slow kinetic effects. Using the calorimeter in the isothermal mode, it has been possible to observe the thermokinetics of gelation as a function of temperature.

It will be possible, from these results, to provide a detailed description and plausible molecular mechanism for the gelation of hemoglobin S. (Ross, Hofrichter, Eaton)

Studies on Polynucleotides

1) Model building studies taken in conjunction with previous diffraction results have strongly indicated that both poly G and poly I have the same four-stranded structure. (Zimmerman)

2) Polynucleotides precipitate as well defined pH values. These pH values are dependent upon both the base composition and the ionic strength of the medium. At low ionic strength, all the polymeric components in solution tend to precipitate together. The pH value at which such co-precipitation occurs can be predicted based upon a simple model which requires charge neutralization between the bases and phosphate residues for insolubility, i.e., precipitation occurs at the net isoelectric point of the solution. Spectrophotometric pH values for some common nucleosides were determined for use in calculating the isoelectric points. At higher ionic strengths, there were instances of fractional precipitation. Also at higher ionic strengths several homopolymers (poly A, poly G, and to a lesser extent, poly U) had a second zone of insolubility near neutral pH values. (Zimmerman)

3) The radius of gyration of polydeoxyadenylate at low temperature, measured by light scattering corresponds to a rod of length 2.85 Å previously observed for polyriboadenylate.

The radius of gyration of polyriboadenylate dissolved in formamide at room temperature is consistent with the presence of a rigid backbone. This appears to demonstrate conclusively that the extended dimensions of polynucleotides which have previously been observed in aqueous solutions are inherent in the backbone and do not depend upon stacking interactions, which are completely abolished in formamide. Furthermore, it is evident that water does not play any special role in such structures. (Felsenfeld, Achter, Stannard)

4) The question of syn-anti rotational isomerism about the glycosidic C-N bond has been one of the most intensively investigated aspects of nucleotide structure in recent years. It has been generally agreed that both syn and anti rotamers of purine nucleosides are capable of existence but that the latter form is favored. Though a number of syn nucleosides have been described, there has been an impasse at the next step in determining what special properties, if any, this less common conformation would confer on polynucleotides. The concensus of published opinion has been that a syn polynucleotide would not be able to form regular helices. A recent semi-empirical energy calculation has predicted as well that syn polynucleotides in general and poly (BrA) in particular would not be able to undergo single-stranded stacking.

The major impediment in this area of structural study has been the lack of any syn homopolymers for experimental investigation. Attempts to polymerize 8-substituted purine nucleoside diphosphates, including 8BrADP, have been uniformly unsuccessful in several laboratories.

A method has now been found to polymerize 8BrADP and has been used to synthesize the first all-syn homopolynucleotide, poly (8BrA). The method requires the use of an oligonucleotide primer, even though the polynucleotide phosphorylase used is primer-independent by the usual criteria. At the end of the reaction the terminal oligo I primer is selectively removed with T_1 ribonuclease. Though the polymerization is very slow the yield of polymer is quite high. The primed polymer has an average chain length of 67 residues of which 63 are BrAMP.

The properties of poly (BrA) are striking in several respects. Contrary to published predictions, the polymer is not only stacked but forms with itself a regular helix of high stability. The hypochromism is the highest yet reported, and temperature profile of absorbance is strongly cooperative. The dependence of Tm on $\log[Na^+]$ is the same as that of most heteroduplex helices. The infrared spectrum of poly (BrA) exhibits a striking splitting of the 1625 cm^{-1} ring vibration into a well-resolved doublet, not previously observed in any other polynucleotide. We have interpreted the results in support of a hydrogen-bonded double helix with a bonding scheme which places the two strands in non-identical environments. (Howard, Frazier, Miles)

5) Previous work in our laboratory has shown that introduction of a 6N-methyl residue into the amino group of poly (A) still permitted formation of a two-stranded Watson-Crick helix. Recent work in collaboration with Prof. Ikehara (Osaka University) and Dr. Hattori (LMB) has shown that when Watson-Crick bonding is blocked (by a 2-Me group), and a 6NMe group is present, a Hoogsteen A·U helix is formed. In this case, however, the 6NMe group is not significantly destabilizing, in marked contrast to the result with Watson-Crick helices. This difference has been interpreted in terms of a restricted rotation of the methylamino group about the C-N bond to the purine, the favored geometrical isomer having 6NMe syn to N1 of the ring. We had previously (Shoup, Becker and Miles, 1972) observed such a restriction in an NMR study of 4NMe cytosine derivatives, and had proposed that the inability of the predominant isomer (Me syn to N3) to hydrogen bond was responsible for the unexpected failure of a polynucleotide containing the 4NMe grouping

to interact with poly (I). In the methylated poly (A) examples, however, both geometrical isomers can form base pairs, the Watson-Crick pair being destabilized by the unfavorable rotational equilibrium of the 6NMe group, while the Hoogsteen pair is unaffected by this substitution. These studies provide basic information for evaluating the base pairing status of methylated adenine residues occurring in DNA in modification-restriction systems. (Miles, Ikehara, Hattori)

6) A number of substituted GMP derivatives have been synthesized with the purpose of deciding between two geometrically isomeric three-stranded $G \cdot C_2$ helices. Though the study is still in progress, the results to date clearly demonstrate that the second poly (C) strand is associated by Hoogsteen pairing rather than the reverse. (Miles, Hattori)

7) Limited and controlled digestion of MS2 RNA by ribonuclease T1 and electrophoretic separation of the RNA fragments by acrylamide gel electrophoresis has been accomplished. Oligonucleotides after ribonuclease T1 complete digestion were analyzed by fingerprinting technique. A rough map of MS2 RNA was made using ribonuclease T1 resistant oligonucleotides as markers. Fifteen large oligonucleotides were classified into three cistrons of the phage. (Fuke)

Protein Structure

1) X-ray diffraction studies of proteolytic enzymes.

a) The refinement of the coordinates of tosyl γ-chymotrypsin at 2.7 A resolution has been continued. Data are being collected to extend the resolution to 2 A. An analysis of the refinement procedure using different computational and computer display techniques is being carried out. Further display techniques have been developed which will enable a large part of the refinement to be carried out by computer with human intervention. (Davies, Cohen)

b) The systematic search for new heavy atom derivatives of the acid protease from Rhizopus chinensis has been continued. Although a number of new derivatives have been discovered, they all bind in the same site as the original lead derivative. Since these derivatives include several mercurials, we are in the process of searching for an additional site. An electron density map at 4 A resolution has been calculated, which permitted the molecular surface to be defined. The molecule is an extended elipsoid with dimensions roughly 40 x 45 x 55 A and is characterized by a large cleft running across the middle of the molecule. The observed heavy atom binding sites occur principally within the cleft. A known inhibitor of the enzyme, 1,2-epoxy-3 (p-nitrophenoxy) propane, has been tested on the crystals. Difference electron density maps show that this inhibitor binds in the cleft of the enzyme. It is proposed to extend these investigations to inhibitors containing a heavy atom so that additional heavy atom derivatives of the protein may be obtained. (Davies, Subramanian, Swan)

2) Protein chemistry of IgM immunoglobulin

The heavy chain of a mouse IgM was found to be similar to human chains

of the same class in molecular weight, carbohydrate content and amino acid content. The heavy chain (α) derived from a mouse myeloma IgA protein has been found to contain 100 fewer amino acid residues than other mouse α chains. Partial sequences of the deleted α chain and a non-deleted α chain indicate that most of the deletion in the former occurs in the COOH-terminal portion of the chain (Robinson)

3) X-ray analysis of the binding site of an immunoglobulin

The crystal structure of the Fab fragment of the mouse myeloma protein McPC 603 has been determined at 3.1 A resolution. The molecule is seen to consist of two chains and each chain is divided into two rather homologous structural units or "domains". The tertiary structure within the domain appears to be very similar for all the myeloma proteins hitherto examined. However, considerable deferences occur in the disposition and spatial location of the hypervariable regions at the terminus of the molecule. In McPC 603 five hypervariable regions combine to form a wedge-shaped cavity approximately 20 x 15 A with a depth of about 12 A. The second hypervariable region of the light chain is excluded from the binding site by a 6 residue insertion in the first hypervariable region of the same chain.

The crystals of McPC 603 have been previously shown to bind phosphoryl-choline. The binding site occurs at the tip of the molecule in the cavity formed by the hypervariable regions. The phosphate is on the surface of the molecule with the choline moiety pointing in towards the center of the molecule. Specific interactions occur between tyrosine 33 and arginine 52 of the heavy chain and the phosphate group to which they appear to be hydrogen bonded. Fewer interactions occur with the residues of the light chain.

The electron density map has permitted a molecular model to be constructed and the coordinates obtained from this model have been used in a computer display system to line up the molecule with another Bence-Jones fragment of an immunoglobulin. Significant departures from three-dimensional homology occur only at the hypervariable loops. (Davies, Segal, Padlan, Cohen, Silverton)

Protein Synthesis in Mammals.

Investigations of the chemical and enzymatic properties of Elongation factor 2, the soluble protein factor required for the translocation step in polypeptide chain elongation have continued. The tryptic peptide containing the site of the inactivation of the factor by NAD^+ and diphtheria toxin has been isolated. The pure peptide consists of 15 amino acid residues whose sequence is Phe-Asp-Val-His-Asp-Val-Thr-Leu-His-Ala-Asp-Ala-Ile-X-Arg. The ADP ribose is linked to the unknown residue, X. X is a weakly basic residue which does not correspond to any amino acid commonly occurring in proteins nor to any of the known basic amino acids occurring less frequently. It is present in unmodified EF-2, possibly to the extent of only one residue per mole of EF-2. (Robinson, Maxwell)

A study of the binding of guanosine nucleotides to EF-2, and the effects of ribosomes on the interaction has been carried out by equilibrium dialysis

and by an improved filter-binding assay. GDP binds tighter than GTP by an order of magnitude (K_d = 4×10^{-7} and 4×10^{-6} respectively). Ribosomes inhibit the binding of GDP but not GTP. One possible interpretation of the data is that the guanosine nucleotides could modulate protein synthesis by affecting the binding of EF-2 to ribosomes and its release therefrom. (Maxwell, Henriksen)

Serial No. NIAMDD-LMB-1
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Genetics and structure of the oncogenic virus, SV40

Previous Serial Number: NIAMDD-LMB-18

Principal Investigator: Robert G. Martin (12/12)

Other Investigators: Dr. Rein Saral (12/12); Dr. Janice Y. Chou (12/12); Dr. Jesus Avila (12/12); Dr. Robert D. Brown (9/12)

Man Years:

Total:	4-9/12
Professional:	4-9/12
Other:	0

Project Description:

Objective: To analyze the genetic structure of the oncogenic virus, SV40, through the isolation of conditional lethal mutants. Interaction between host functions and viral replication will also be investigated.

Major Findings: (a) Seventy six temperature sensitive mutants of SV40 have been isolated (Chou & Martin). In order to carry out complementation analyses on this large group of mutants a new technique was developed which is applicable to all animal viruses. Using this technique it was possible to perform complete analyses on all the mutants and the results clearly indicate that the mutants can be divided into four complementation groups, A, B, C, and D and a fifth group, BC, which fails to complement with both B and C mutants.

(b) Characterization of viral DNA synthesis in the mutants at the restrictive temperature has been undertaken (Chou, Avila & Martin). The mutants can be divided into "early mutants", those which fail to synthesis viral DNA at the restrictive temperature, classes A and D, and "late mutants", those which can synthesize viral DNA, classes B, C, and BC. It has been shown that A function is required continuously for viral DNA synthesis to proceed at the restrictive temperature, i.e., preincubation at the permissive temperature followed by a shift to the restrictive temperature results in shut off of the viral DNA synthesis. Furthermore, the shut off has been shown to result from the inability to start new rounds of viral DNA replication since replicative intermediate forms do continue to replicate to form mature DNA molecules.

The block in D mutants appears to precede the establishment of viral DNA synthesis. Incubation at the permissive temperature, followed by shift to restrictive conditions does not shut off subsequent viral DNA synthesis provided the incubation has proceeded at the permissive temperature for at least 10 to 20 hours. Yet at 20 hours at the permissive temperature viral DNA synthesis has not yet begun.

(c) A large number of requests have already been received for these mutants and as a result of their dissemination we have learned that D function is required for the enhancement of adenoviruses replication (MaryAnn Jerkofsky, unpublished results) and physical mapping of the mutants has been partially completed (Daniel Nathans, unpublished results).

(d) Since D mutants fail to carry out any known viral function it was necessary to determine whether early mRNA is synthesized by these mutants. Preliminary results (Saral) indicate that early mRNA is made.

(e) Transformation studies using Chinese hamster cells have been initiated (Martin, Chou, Avila, & Saral). Preliminary results suggest that only A function is required for either the establishment or maintenance of the transformed state. The implication of this finding is that a biochemical understanding of the A function will lead to a biochemical understanding of the oncogenic process.

Significance to bio-medical research and the program of the institute: The significance of these projects is that they may lead to some insights into carcinogenesis and other metabolic diseases.

Publications:

Chou, Janice Y. and Martin, Robert G.: Complementation analysis of SV40 mutants. Journal of Virology (in press).

Chou, Janice Y., Avila, J., and Martin, R.G.: DNA synthesis in temperature sensitive mutants of simian virus 40. Journal of Virology (in press).

Serial No. NIAMDD-LMB-2
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Genetics and physiology of bacteriophage P1

Previous Serial Number: NIAMDD-LMB-20

Principal Investigator: J. L. Rosner (12/12)

Collaborator: Michael M. Gottesman (A:LMB)

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: An understanding of the mechanisms which regulate vegetative growth, plasmid formation, transduction and gene expression by the temperate bacteriophage P1. Special attention has been devoted to:

a) The isolation and characterization of P1 prophages which carry bacterial genes and which are deleted for P1 genes.

b) The structure of λCM (with Michael Gottesman).

Major Findings: a) Among variants of P1 carrying bacterial *pro* (proline-biosynthesis) genes previously described, one type has been intensively studied. This P1-*pro* prophage exists as a circular plasmid consisting of (a) a full complement of P1 genes starting with the gene 2 region, (b) a duplication of the gene 2 region and (c) the bacterial *pro* genes. Thus, a gene 2 region occurs at both junctions of phage and bacterial DNA.

When the prophage is induced, its DNA is replicated and packaged. The entire structure is too large to be packaged within a single P1 phage. However, the encapsidation of P1 DNA including both gene 2 regions can occur, resulting in a normal P1 (*pro*$^-$) phage. The reciprocal event, the encapsidation of the bacterial *pro* genes, bordered on both sides by gene 2 regions, results in the production of *pro*$^+$ transducing particles.

The *pro*$^+$ transductants produced by infection with the latter type particle carry the gene 2 region but no other P1 genes. Preliminary mapping experiments indicate that the *pro*$^+$ genes in these transductants are located on the *E. coli* chromosome near the *his* region (quite distant from the normal

pro location). The possibility that the gene 2 region may be responsible for promoting recombinations between P1 and the host chromosome is being investigated.

b) The structure of λCM. The structure of λCM, a bacteriophage λ variant which carries a determinant for resistance to chloramphenicol, has been elucidated. Heteroduplex mapping shows that the CM determinant is inserted into the λ chromosome within the b2 region. The insertion is about 5% the size of λ or about 1.7 million daltons.

The CM determinant is lost from λCM when grown vegetatively at a frequency of about 2% per λ per cycle of growth. However, the loss is not complete; portions of the original insertion remain as observed both by heteroduplex mapping and CsCl equilibrium centrifugation. The recombinational mechanism responsible for this loss of CM is being investigated.

Significance to bio-medical research and the program of the institute:

Knowledge of the interaction of virus and host cell is important for an understanding of a variety of diseases. Insofar as this research contributes to our understanding of these interactions as they occur in bacteria, it will have significance to the objectives of this institute.

Serial No. NIAMDD-LMB-3
1. Laboratory of Molecular Biology
2. Microbial Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The processes of the establishment of the oncogenic genes after the infection of tumor viruses

Previous Serial Number: None

Principal Investigator: Toshiya Takano

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: An understanding of the processes of the establishment of the oncogenic genes after the infection of tumor viruses. Specifically, attention has been focused on the formation of intermediates of RNA tumor viruses, which leads to the integration of the viral genomes on the host chromosome.

Major Findings: The above objectives are aimed by studying the fate of the genome RNA of Murine leukemia virus after infection of cultured cells. DNA-RNA hybrid molecules in cultured cells infected by murine leukemia viruses were analyzed. Purified ^{3}H-Uridine labelled Rauscher leukemia virus, SLV9, was infected with mouse embryo fibroblasts or normal rat kidney cells. At various times after infection, cells were separated into nuclear and cytoplasmic fractions by treatment with sodium deoxycholate-Tween-40 and nucleic acid extracted by SDS-phenol-chloroform treatment. All radioactivity recovered was acid insoluble and alkaline sensitive. Between 45 to 70 min after infection, a transient and synchronized shift of the radioactive peak toward the RNA-DNA hybrid region occurred in both the nuclear and the cytoplasmic fractions. The density of the cytoplasmic hybrid shifted to 1.56 (RNA-about 50%) while the sedimentation rate decreased by 36S to 14S. However, the density of the nuclear hybrid shifted from 1.58 to 1.48 (RNA=57 to 17% respectively) while its sedimentation rate remained at about 61S. Both of the hybrids in the nuclear and the cytoplasmic fractions showed still hybrid density after heat denaturation. A model is proposed here: In the early stages of infection, the viral RNA is translocated into the nucleus, with conservation of the whole 70S viral RNA genome in an RNA-DNA hybrid molecule. In the nucleus, DNA synthesis proceeds on these hybrids leading to the formation of a double stranded "viral" DNA and allowing the integration of the viral information. Alternatively, the RNA-DNA hybrid could be integrated

directly. The inhibition of the formation of these hybrids by various inhibitors to DNA synthesis, and hybridization of these hybrids with viral and the host DNA are now under investigation.

Significance to bio-medical research and the program of the institute: Viruses induce a series of new biological processes in the infected host cells. Specially, a group of viruses called tumor viruses cause various cancers and tumors by the integration of viral genomes into the host chromosome and the expression of a series of viral gene functions. To understand the processes of the integration of the viral genome on the host chromosome at a molecular and enzymological level, is an important goal for the future control of these viral processes including the infection and oncogenesis by animal viruses.

Publications:

1. Takano, T. and Hatanaka, M.: DNA-RNA hybrid found in cells infected by Murine leukemia virus. Cold Spr. Harb Symp Quant Biol., Vol. 39 (in press).

Serial No. NIAMDD-LMB-4
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Enzymatic Joining of DNA Strands and its Role in Genetic Recombination; In vivo and in vitro studies on DNA replication and recombination

Previous Serial Number: NIAMDD-LMB-3

Principal Investigator: Martin Gellert

Other Investigators: Michael M. Gottesman
Kiyoshi Mizuuchi
Sue Wickner (Guest Worker)

Man Years:

Total:	3 3/4
Professional:	2 3/4
Other:	1

Project Description:

Objectives: To understand the physiological role of DNA ligases; to study the biochemical mechanisms of DNA replication and recombination.

Methods: Ultracentrifugation, standard enzymological and microbiological techniques, and electron microscopy.

Major Findings: 1.) DNA ligase mutants of E. coli (M. M. Gottesman, M. Gellert).

We have studied two lig mutants, extracts of which contain no assayable ligase at 42°. Paradoxically, both mutants are able to carry out UV repair and recombination, processes presumed to require DNA ligase, and both are able to covalently circularize linear λ phage DNA in vivo at 42°. Both of these lig mutants accumulate Okazaki fragments, believed to be intermediates in discontinuous DNA replication, but only one of these lig mutants fails to grow at 42°. By genetically introducing a lop mutation which increases by a factor of five the number, but not type, of molecules of defective DNA ligase in the lig mutants, we have shown that both mutants produce small amounts of ligase at 42°, i.e., they are not absolutely defective. The small increase in defective ligase in the inviable mutant is sufficient to allow growth at 42°. Thus, the paradoxical behavior of these mutants can still be accounted for by postulating that lig is the only locus for DNA ligase in E. coli, but that the processes of replication, repair and recombination of DNA in this organism require differing amounts of DNA ligase.

We had previously shown that the initial rate of DNA synthesis in lig mutants was the same as the rate of DNA synthesis in wild-type E. coli, and have been characterizing the nature of the DNA synthesized in lig mutants. By constructing double mutants of lig with known DNA-ts mutations (dnaA lig and dnaG and lig), we have been able to show that the DNA synthesis observed in lig mutants does not occur in the absence of normal bacterial DNA replication.

2.) Studies on "reverse" mutants of phage λ (M. M. Gottesman, K. Mizuuchi). The recombination proficient λ phage variant known as "reverse" was derived from a λ phage deleted for recombination functions. We have shown that this phage carries a substitution of DNA recombination genes derived from the E. coli chromosome. Studies in collaboration with A. J. Clark indicate that this phage carries the gene or genes coding for the RecE recombination pathway in E. coli, including the gene which codes for the newly discovered Exonuclease VIII. Work in progress indicates that the genes for this recombination pathway are derived from a previously undetected defective prophage.

3.) In vitro studies on the site-specific recombination system (Int) of phage λ (K. Mizuuchi).

a) Isolation of bacterial mutants unable to integrate phage λ: An E. coli mutant which gives rise to λ lysogens at reduced frequency following infection has been isolated. This strain is capable of supporting Int-mediated recombination at a normal frequency in phage by phage crosses, suggesting that the strain has an altered attachment site for λ. The further characterization of this strain and a search for other mutant strains, perhaps lacking a host function necessary for integration, is in progress.

b) In vitro Int-promoted recombination: We have previously shown that λ Int protein (integrase) can be radiochemically purified from λ-infected E. coli (Mizuuchi, unpublished), but no biological activity has yet been demonstrated for this protein in vitro. Development of an assay which will detect in vitro Int-promoted recombinants is in progress.

4.) Studies on E. coli DNA replication in vitro (S. Wickner, guest worker).

The conversion of ϕX174 single-stranded DNA to duplex DNA is catalyzed by crude extracts of E. coli in a reaction dependent on the products of the dnaB, C(D), E (DNA polymerase III) and G genes and not dependent on the product of the dnaA gene, DNA polymerase I, DNA polymerase II, or RNA polymerase. The involvement of these gene products in this reaction was shown by the increased thermolability of ϕX174 DNA-dependent DNA synthesis in extracts from dna temperature sensitive cells when compared to extracts from temperature resistant revertant cells. Thus the stimulation of inactive crude extracts by fractions from wild-type cells provided complementation assays for the purification of the dna B, C(D), E and G gene products. The identity of proteins isolated by these assays has been established by the isolation of dna B, C (D), E and G gene products from dna B, C, E, and G temperature sensitive cells, respectively, and the demonstration in each case that the temperature sensitive gene product was more thermolabile than the wild-type product. The dna B, C (D) and G gene

products have been partially characterized. DNA-dependent and independent ribonucleoside triphosphatase activities are part of or closely associated with the dna B gene product. To date no other enzymatic activities can be attributed to these proteins.

The isolation of all of the proteins required for this reaction is in progress. The mixture of purified dna B, C (D), E and G gene products alone does not catalyze detectable DNA synthesis in the presence of ϕX174 DNA. Fractionation of the crude extract yields three protein fractions each of which is required in addition to purified dna B C (D), E and G gene products for ϕX174 DNA-dependent deoxynucleotide incorporation. Using this defined in vitro DNA replicating system, the properties of these proteins, their interactions with each other, nucleoside triphosphates, and DNA are being studied to determine their roles in DNA replication. This work is being done in collaboration with J. Hurwitz.

Publications:

Gottesman, M., Hicks, M. and Gellert, M.: Genetics and Function of DNA Ligase in Escherichia coli. J. Mol. Biol. : 77, 531-547, 1973.

Serial No. NIAMDD-LMB-5
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Protein Synthesis in Mammals: Mechanisms and Metabolic Controls

Previous Serial Number: NIAMDD-LMB-4

Principal Investigator: Elizabeth S. Maxwell

Other Investigators: Elizabeth Robinson
Ole Henriksen
Sharon Krauss

Man Years:
Total: 3 1/3
Professional: 3 1/3

Project Description:

Objectives: Our overall objective is to gain an understanding of the mechanisms and metabolic controls of protein synthesis in mammals. For the past few years we have worked toward obtaining a partial understanding of these processes through detailed knowledge of the soluble enzymes involved in polypeptide chain formation. Work in this area is continuing, but we are now expanding our interest to include translation of specific messenger RNAs in crude systems.

Methods Employed: All of the methods employed are either standard procedures or minor modifications thereof.

Major Findings: Investigations of the chemical and enzymatic properties of Elongation factor 2, the soluble protein factor required for the translocation step in polypeptide chain elongation have continued. The tryptic peptide containing the site of the inactivation of the factor by NAD^+ and diphtheria toxin has been isolated. The pure peptide consists of 15 amino acid residues whose sequence is Phe-Asp-Val-His-Asp-Val-Thr-Leu-His-Ala-Asp-Ala-Ile-X-Arg. The ADP ribose is linked to the unknown residue, X. X is a weakly basic residue which does not correspond to any amino acid commonly occurring in proteins nor to any of the known basic amino acids occurring less frequently.

It is present in unmodified EF-2, possibly to the extent of only one residue per mole of EF-2.

A study of the binding of guanosine nucleotides to EF-2, and the effects

Serial No. NIAMDD-LMB-6
1. Laboratory of Molecular Biology
2. Metabolic Enzymes
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structural Studies on Mouse Myeloma Immunoglobulins

Previous Serial Number: (none)

Principal Investigator: Elizabeth A. Robinson

Cooperating Units: Dr. Ettore Appella, NCI
Dr. K. Robert McIntire, NCI

Man Years:
Total: 1/3
Professional: 1/3

Project Description:

Objectives: 1) Chemical characterization of heavy chains derived from mouse myeloma immunoglobulin M (IgM) proteins and their comparison to heavy chains derived from human IgM proteins. 2) Determination of the primary structure of two heavy chains from mouse IgA proteins, one of which is deleted.

Major Findings: The heavy chain of a mouse IgM was found to be similar to human chains of the same class in molecular weight, carbohydrate content and amino acid content.

The heavy chain (α) derived from a mouse myeloma IgA protein has been found to contain 100 fewer amino acid residues than other mouse α chains. Partial sequences of the deleted α chain and a non-deleted α chain indicate that most of the deletion in the former occurs in the COOH-terminal portion of the chain.

Significance to NIAMDD Research: The elucidation of the structure of mouse myeloma immunoglobulins is pertinent to the understanding of the structure and specificity of human antibodies.

Proposed Course: To determine the exact nature and location of the deletion in the mouse α chain (described above) and to determine the genetic event causing the deletion.

Publications:
Robinson, Elizabeth A., Appella, Ettore, and McIntire, K. Robert: Structural Studies of a Heavy Chain from a Mouse Immunoglobulin M with Antibody Activity. The Journal of Biological Chemistry: 248, 7112-7117, 1973.

Serial No. NIAMDD-LMB-7
1. Laboratory of Molecular Biology
2. Section on Molecular Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Replication, Recombination and Repair of Microbial DNA

Previous Serial Number: NIAMDD-LMB-6

Principal Investigator: J. Tomizawa (12/12)

Other Investigators: Y. Sakakibara (5/12)
Y. Sugino (12/12)
A. C. Satterthwait (12/12)

Man Years:

Total:	3-5/12
Professional:	3-5/12
Other:	0

Project Description:

Objectives: Molecular biological understanding of the mechanisms of replication, recombination and repair of DNA is aimed.

Methods Employed: The mechanisms are studied mainly with E. coli and phages. Various genetic tricks are used extensively. Density gradient centrifugation (CsCl and sucrose), differential labeling of DNA with radioisotopes and heavy isotopes were used for isolation and analysis of DNA. Structures of DNA were visualized by the electron-microscopic method.

Major Findings:

Replication of colicin E1 plasmid DNA in cell extracts. DNA synthesis in vitro has been observed in several systems but a system in which the whole process of replication of double-stranded DNA is carried out has not been established. Such a system should show, at least, initiation of DNA synthesis at a specific site, continuation of replication in a semiconservative manner and formation of progeny molecules by segregation. We established a soluble system in which closed-circular DNA molecules of colicin E1 plasmid initiate and complete a round of semiconservative replication.

Cell extracts were prepared from E. coli carrying colicin E1 plasmid. The DNA in extracts was almost exclusively closed-circular Col E1 DNA. Labeled deoxyribonucleotides were incorporated into DNA in extracts. Col E1 DNA was the sole DNA product, and was composed of completely replicated mole-

cules and a class of replicative intermediates. The intermediates carried an average of approximately two pieces of DNA fragments which were approximately 6S and were not covalently attached to the parental DNA strands. The replication was initiated on closed-circular molecules and the complete molecules were synthesized semi-conservatively. The DNA synthesis depended on the four ribonucleoside triphosphates and was sensitive to rifampicin. A round of DNA replication, once initiated, was completed in the presence of rifampicin indicating that RNA synthesis is involved in the initiation of Col E1 DNA replication. Most of the replicated molecules were isolated in super-coiled structures. These results indicate that this soluble system is capable of carrying out the entire processes of Col E1 DNA replication.

The addition of 10% glycerol and/or 2 mM spermidine to cell extracts blocks synthesis of completely replicated molecules while enhancing synthesis of molecules containing newly synthesized 6S DNA fragments. These molecules are early replicative intermediates. Their synthesis is sensitive to rifampicin and depends on RNA synthesis. RNA components are linked to the 6S DNA molecules.

The structures of molecules of colicin E1 plasmid DNA that were in the process of being replicated in vitro were examined electron microscopically. Circular molecules containing a loop of 7% of the length of the molecules were the major class of replicating molecules. The location of the loop was determined by treating with restriction endonuclease EcoR1 which introduced one unique double-strand break in the colicin E1 plasmid DNA molecules. The loops had a specific location with two branch points at approximately 17 and 24% of the molecular length from the endonuclease sensitive site. Molecules with a larger loop were observed with a preparation labeled with 5-bromodeoxyuridine and enriched for these molecules. One of the branch points in these molecules was located at approximately 17% from the endonuclease sensitive site independent of the size of the loops. These results indicate that the origin of replication of the plasmid DNA is located within the small loop and the replication proceeds unidirectionally. The molecules that had completed a round of replication had the monomeric twisted circular structure.

Significance to Bio-medical Research and the Program of the Institute: Understanding of the mechanisms of replication, recombination and repair has prime importance in genetics. Extensive investigation can be made with the system with which we are working. Understanding of these processes will have significance for bio-medical research and the program of the Institute.

Proposed Course of Project: Studies on the mechanisms of in vitro DNA replication and those of genetic recombination will be continued.

Publications:

Sakakibara, Y., and Tomizawa, J.: Replication of Colicin E1 Plasmid DNA in Cell Extracts. Proc. Nat. Acad. Sci. USA 71: 802-806, 1974.

Sakakibara, Y., and Tomizawa, J.: Replication of Colicin El Plasmid DNA in Cell Extracts: II. Selective Synthesis of Early Replication Intermediates. Proc. Nat. Acad. Sci. USA 71: 1403-1407, 1974.

Serial No. NIAMDD-LMB-8
1. Laboratory of Molecular Biology
2. Section on Molecular Genetics
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Base Sequence Analysis of MS2 Phage RNA

Previous Serial Number: NIAMDD-LMB-7

Principal Investigator: M. Fuke (12/12)

Man Years:

Total:	12/12
Professional:	12/12
Other:	0

Project Description:

Objectives: Determination of the complete base sequence of MS2 phage RNA is aimed through the development of a method to obtain unique collections of fragments of the RNA.

Methods Employed: Limited and controlled digestion of MS2 RNA by ribonuclease T1 and electrophoretic separation of the RNA fragments by acrylamide gel electrophoresis. Oligonucleotides after ribonuclease T1 complete digestion were analyzed by fingerprinting technique.

Major Findings: A rough map of MS2 RNA was made using ribonuclease T1 resistant oligonucleotides as markers. Fifteen large oligonucleotides were classified into three cistrons of the phage.

Significance to Bio-medical Research and the Program of the Institute: Development of a method which is widely applicable for specific fragmentation of nucleic acids has fundamental importance for nucleic acid chemistry and biology. Sequence analysis of MS2 RNA will allow us to explain various functions of the phage based on RNA structure. This research will increase fundamental knowledge of bio-medical knowledge and help to understand RNA oncogenic viruses which carry small single stranded RNA as MS2 phage carries.

Proposed Course of Project: This research will be continued. Further fractionation will be performed and the ends newly formed by ribonuclease T1 will be analyzed.

Publications:

Fuke, M.: Introduction of Specific Cleavages into RNAs of RNA Bacteriophages for Determination of Base Sequences. Proc. Nat. Acad. Sci. USA 71: 742-745, 1974.

Serial No.: NIAMDD-LMB-9
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Crystal structure investigation of antigen antibody interaction

Previous Serial Number: NIAMDD-LMB-1

Principal Investigators: David R. Davies, D. M. Segal, E. A. Padlan, G. H. Cohen and E. W. Silverton

Cooperating Units: Dr. Michael Potter, NCI and Dr. Stuart Rudikoff, NCI

Man Years:

Total:
Professional:
Others: 0

Project Description:

Objectives: A comparative investigation of the crystal structures of the Fab fragment of immunoglobulin molecules that bind strongly to several different antigens. In this way, it is hoped to map out the binding site of antibodies and to illustrate the mechanism of antigen-antibody interaction.

Methods Employed: Preparation and analysis of pure materials. Crystallization and x-ray diffraction analysis with the heavy atom isomorphous replacement method.

Major Findings: The crystal structure of the Fab fragment of the mouse myeloma protein McPC 603 has been determined at 3.1 A resolution. The molecule is seen to consist of two chains and each chain is divided into two rather homologous structural units or "domains". The tertiary structure within the domain appears to be very similar for all the myeloma proteins hitherto examined. However, considerable deferences occur in the disposition and spatial location of the hypervariable regions at the terminus of the molecule. In McPC 603 five hypervariable regions combine to form a wedge-shaped cavity approximately 20 x 15 A with a depth of about 12 A. The second hypervariable region of the light chain is excluded from the binding site by a 6 residue insertion in the first hypervariable region of the same chain.

The crystals of McPC 603 have been previously shown to bind phosphorylcholine. The binding site occurs at the tip of the molecule in the cavity formed by the hypervariable regions. The phosphate is on the surface of the molecule with the choline moiety pointing in towards the center of the molecule.

Specific interactions occur between tyrosine 33 and arginine 52 of the heavy chain and the phosphate group to which they appear to be hydrogen bonded. Fewer interactions occur with the residues of the light chain.

The electron density map has permitted a molecular model to be constructed and the coordinates obtained from this model have been used in a computer display system to line up the molecule with another Bence-Jones fragment of an immunoglobulin. Significant departures from three-dimensional homology occur only at the hypervariable loops.

Significance to Bio-medical Research and the Program of the Institutes: An understanding of the molecular basis of interaction between antibody and antigen is fundamental to our understanding of the immune response.

Proposed Course: We plan to complete the structural determination of McPC 603 Fab and commence the analysis of J539. In addition, we plan to make comparisons between the 603 structure and that of other immunoglobulin molecules.

Publications:

Padlan, E. A., Segal, D. M., Spande, T. F., Davies, D. R., Rudikoff, S. and Potter, M.: Structure at 4.5 A resolution of a phosphorylcholine-binding Fab. Nature 245, 165-166, 1973.

Padlan, E. A., Segal, D. M., Cohen, G. H., Davies, D. R., Rudikoff, S. and Potter, M.: The immune system: genes, receptors, signals. In Proceedings of the 1974 I.C.N.-UCLA Symposium on Molecular Biology, in press.

Serial No.: NIAMDD-LMB-10
1. Laboratory of Molecular Biology
2. Section on Molecular Structure
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: X-ray diffraction investigation of proteolytic enzymes

Previous Serial Number: NIAMDD-LMB-2

Principal Investigators: David R. Davies, E. Subramanian, Gerson H. Cohen and Ian D. A. Swan

Man Years:

Total: 2-1/4
Professional: 2-1/4
Others: 0

Project Description:

Objective: The determination of the 3-dimensional structure of proteolytic enzymes, in particular, the structure of gamma chymotrypsin and of the acid protease from Rhizopus chinensis.

Methods Employed: 1. The refinement of the coordinates of tosyl γ-chymotrypsin at 2.7 A resolution has been continued. Data are being collected to extend the resolution to 2 A. An analysis of the refinement procedure using different computational and computer display techniques is being carried out. Further display techniques have been developed which will enable a large part of the refinement to be carried out by computer with human intervention.

2. The systematic search for new heavy atom derivatives of the acid protease from Rhizopus chinensis has been continued. Although a number of new derivatives have been discovered, they all bind in the same site as the original lead derivative. Since these derivatives include several mercurials, we are in the process of searching for an additional site. An electron density map at 4 A resolution has been calculated, which permitted the molecular surface to be defined. The molecule is an extended elipsoid with dimensions roughly 40 x 45 x 55 A and is characterized by a large cleft running across the middle of the molecule. The observed heavy atom binding sites occur principally within this cleft. A known inhibitor of the enzyme, 1,2-epoxy-3 (p-nitrophenoxy) propane, has been tested on the crystals. Difference electron density maps show that this inhibitor binds in the cleft of the enzyme. It is proposed to extend these investigations to inhibitors containing a heavy atom so that additional heavy atom derivatives of the protein may be obtained.

Significance to Bio-medical Research and the Program of the Insitute: The work on the mode of binding of specific inhibitors to chymotrypsin has

turned out to have fairly universal application and has been used to show how trypsin and trypsin inhibitor interact. It has also thrown light on the evolutionary mechanism for the production of enzymes. The acid protease with its peak of activity around pH 3.5 is very similar in its catalyic mechanism to other enzymes of this class, i.e. pepsin, rennin (chymosin), and the cathepsins. X-ray diffraction studies are being carried out on both rennin and pepsin, and also on a mold enzyme from penicillium. A detailed knowledge of the structure of this enzyme and its mechanism of catalysis should be of use in predicting the properties of the enzymes found in lysosomes, and comparison of this structure with those of the other enzymes should throw considerable light on the evolutionary mechanism for this class of enzyme.

Proposed Course of Project: Find the precise location of the amino acids involved in catalysis. In addition, the search for suitable heavy atom derivatives will be continued. Since the sequence of this enzyme although currently under investigation, is not yet available, the highest resolution will be required in order to assist in obtaining an interpretable electron density map.

Publications: None.

Serial No. NIAMDD-LMB-11
1. Laboratory of Molecular Biology
2. Section on Organic Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Chemical and Structural Investigations of Nucleic Acids and Related Substances

Previous Serial Number: NIAMDD-LMB-5

Principal Investigator: H. Todd Miles

Other Investigators: Dr. F. B. Howard, Dr. M. Hattori, and Mr. J. Frazier

Cooperating Units: Prof. M. Ikehara (University of Osaka)

Man Years:

Total:	4-1/2
Professional:	3-1/2
Other:	1-1/2

Project Description:

Objectives: Basic chemical, biochemical, and structural studies of nucleic acids and related substances.

Methods: Infrared spectroscopy, nuclear magnetic resonance spectroscopy, chemical synthesis and structure elucidation, optical rotation, ultraviolet spectroscopy, use of enzymes for synthesis and structure study.

Major Findings:

The question of syn-anti rotational isomerism about the glycosidic C-N bond has been one of the most intensively investigated aspects of nucleotide structure in recent years. It has been generally agreed that both syn and anti rotamers of purine nucleosides are capable of existence but that the latter form is favored. Though a number of syn nucleosides have been described, there has been an impasse at the next step in determining what special properties, if any, this less common conformation would confer on polynucleotides. The concensus of published opinion has been that a syn polynucleotide would not be able to form regular helices. A recent semi-empirical energy calculation has predicted as well that syn polynucleotides in general and poly (BrA) in particular would not be able to undergo single-strand stacking.

The major impediment in this area of structural study has been the lack of any syn homopolymers for experimental investigation. Attempts to polymerize

8-substituted purine nucleoside diphosphates, including 8BrADP, have been uniformly unsuccessful in several laboratories.

We have now found a method to polymerize 8BrADP and have used it to synthesize the first all-syn homopolynucleotide, poly (8BrA). The method requires the use of an oligonucleotide primer, even though the polynucleotide phosphorylase used is primer-independent by the usual criteria. At the end of the reaction the terminal oligo I primer is selectively removed with T_1 ribonuclease. Though the polymerization is very slow the yield of polymer is quite high. The primed polymer has an average chain length of 67 residues, of which 63 are BrAMP.

The properties of poly (BrA) are striking in several respects. Contrary to published predictions, the polymer is not only stacked but forms with itself a regular helix of high stability. The hypochromism is the highest yet reported, and temperature profile of absorbance is strongly cooperative. The dependence of Tm on log[Na^+] is the same as that of most heteroduplex helices. The infrared spectrum of poly (BrA) exhibits a striking splitting of the 1625 cm^{-1} ring vibration into a well-resolved doublet, not previously observed in any other polynucleotide. We have interpreted the results in support of a hydrogen-bonded double helix with a bonding scheme which places the two strands in non-identical environments. (Howard, Frazier, and Miles)

Previous work in our laboratory had shown that introduction of a 6N-methyl residue into the amino group of poly (A) still permitted formation of a two-stranded Watson-Crick helix. Recent work in collaboration with Prof. Ikehara (Osaka University) and Dr. Hattori (LMB) has shown that when Watson-Crick bonding is blocked (by a 2-Me group), and a 6NMe group is present, a Hoogsteen A·U helix is formed. In this case, however, the 6NMe group is not significantly destabilizing, in marked contrast to the result with Watson-Crick helices. We have interpreted this difference in terms of a restricted rotation of the methylamino group about the C-N bond to the purine, the favored geometrical isomer having 6NMe syn to N1 of the ring. We had previously (Shoup, Becker and Miles, 1972) observed such a restriction in an NMR study of 4NMe cytosine derivatives, and had proposed that the inability of the predominant isomer (Me syn to N3) to hydrogen bond was responsible for the unexpected failure of a polynucleotide containing the 4NMe grouping to interact with poly (I). In the methylated poly (A) examples, however, both geometrical isomers can form base pairs, the Watson-Crick pair being destabilized by the unfavorable rotational equilibrium of the 6NMe group, while the Hoogsteen pair is unaffected by this substitution. These studies provide basic information for evaluating the base pairing status of methylated adenine residues occurring in DNA in modification-restriction systems.

Dr. Hattori has synthesized a number of substituted GMP derivatives with the purpose of deciding between two geometrically isomeric three-stranded $G \cdot C_2$ helices. Though the study is still in progress, the results to date clearly demonstrate that the second poly (C) strand is associated by Hoogsteen pairing rather than the reverse.

Publications:

Ishikawa, F., Frazier, J., and Miles, H. T.: Poly(2-dimethylaminoadenylic acid). Synthesis and characterization of the homopolymer. Biochemistry 12: 4790-4798, 1973.

Howard, F. B., Frazier, J., and Miles, H. T.: Poly 8-bromoadenylic acid: An all-syn homopolynucleotide with a regular, helical structure. J. Biol. Chem., 1974, in press.

Hattori, M., Ikehara, M., and Miles, H. T.: Poly 2-methyl-N6-methyladenylic acid: Synthesis, properties and interaction with polyuridylic acid. Biochemistry, 1974, in press.

Serial No. NIAMDD-LMB -12
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure of Single Strand Polynucleotides in Solution

Previous Serial Number: 8

Principal Investigator: Gary Felsenfeld

Other Investigators: Eugene Achter and Bethel Stannard

Man Years:

Total: 3/8
Professional: 3/8
Others: 0

Project Description:

Objectives: To elucidate the structure of single strand polynucleotides in solution.

Methods Employed: Theoretical analysis, light scattering, viscometry, spectrophotometry, sedimentation velocity, sedimentation equilibrium.

Major Findings: We have studied the properties of polydeoxyadenylate at low temperature, and find that the radius of gyration measured by light scattering corresponds to a rod of length 2.85 Å per nucleotide, compared with the value of 3.2 Å we have previously observed for polyriboadenylate.

We have also measured the radius of gyration of polyriboadenylate dissolved in formamide, and find that at room temperature the coil dimensions are consistent with the presence of a rigid backbone. This appears to demonstrate conclusively that the extended dimensions of polynucleotides which we have previously observed in aqueous solutions are inherent in the backbone and do not depend upon stacking interactions, which are completely abolished in formamide. Furthermore, it is evident that water does not play any special role in such structures.

Significance to Bio-medical Research and the Program of the Institute: This work continues and brings close to completion our understanding of the nature of single strand polynucleotide structures in solution, the relative roles of base stacking, backbone rotational restrictions, and solvent interactions. The conformation of such molecules is a major determinant in their chemical behavior, and this behavior plays a dominant role in the reactivity of naturally occurring RNA, such as messenger RNA, viral RNA, ribosomal and

transfer RNA, under natural conditions in the living cell.

Proposed Course of Project: The major goals of this project appear to have been met, at least for the present, and it is likely that it will be terminated after publication of recently obtained results.

Serial No. NIAMDD-LMB -13
1. Laboratory of Molecular Biology
2. Section on Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Chromatin Structure and Function

Previous Serial Number: 9

Principal Investigator: Gary Felsenfeld

Other Investigators: Richard Axel, William B. Melchior Jr., Bethel Stannard and Barbara Sollner-Webb

Man Years:

Total: 3-3/4
Professional: 3-3/4
Others: 1

Project Description:

Objectives: The object of this project is to determine the structure of chromatin and the relationship of this structure to its biological role in cell differentiation.

Methods Employed: Spectrophotometry, analytical ultracentrifugation, dialysis equilibrium, and other standard techniques are used to study the binding of charged molecules to DNA, and the composition of the regions of DNA to which these molecules are bound. Standard chromatographic and electrophoretic techniques are used in purification and identification of histones. RNA polymerase is purified by classical enzymological techniques and its activity examined by membrane filtration, sucrose gradient sedimentation, or radioisotope incorporation. DNA-RNA hybridization methods are used to detect presence of specific polynucleotide sequences.

Major Findings: We have continued our studies of globin messenger RNA synthesis *in vitro*. We showed last year for the first time that chromatin isolated from duck reticulocytes can serve as a template for the production of globin RNA *in vitro* when transcribed with RNA polymerase from *E. coli*. Chromatin isolated from tissues not producing globin message *in vivo* do not serve as a template for its production *in vitro*. Globin message is detected by hybridization to a globin-specific radioactive DNA probe which is complementary to the globin message sequence. We have extended these observations to a quantitative comparison of the template activity of chromatin from duck reticulocytes and erythrocytes. The former produces globin RNA to the extent of about one part in 10^4 when transcribed *in vitro*. The latter is almost completely inactive as a template, as expected for chromatin isolated from a

tissue not actively producing globin message. In the course of this work we have developed methods of analyzing DNA-RNA hybridization data quantitatively even for reactions in which the globin RNA sequence is not present in excess over the radioactive DNA probe. This permits us to determine that our erythrocyte chromatin preparation produces globin RNA in an abundance about one seventh that produced by reticulocyte chromatin.

We have previously shown that about half the DNA of chromatin is accessible to a variety of chemical probes, including the enzyme staphylococcal nuclease, and the polycation polylysine. We have made use of this reactivity to separate chromatin into two distinct classes of double stranded regions, which we call "covered DNA" and "open DNA". We now ask whether the globin sequence is present in both of these classes of DNA isolated from reticulocyte chromatin. We find that if we anneal open and covered reticulocyte DNA with globin DNA probe, the covered DNA anneals with about 20% more of the probe than does open DNA. We are able to show that the difference arises because a portion of the globin probe sequence is absent from open regions, and we conclude that a portion of the actively transcribed globin gene is not contained in the open region, and is therefore covered by protein. In contrast, the open and covered DNA regions isolated from erythrocyte chromatin show no such difference, suggesting that the presence of the protein covered region is connected in some way with active transcription of globin sequences.

We have shown that chromatin contains the information for tissue-specific transcription. It is possible to show that the information for specific reassembly is also present. If reticulocyte chromatin is dialyzed into solvents containing high concentrations of salt and urea, the proteins are dissociated from the DNA and may be isolated. If these proteins are now added to a purified preparation of duck DNA, and the mixture dialyzed back into low salt, the reconstituted chromatin is a template for globin message production, with an efficiency close to that of the starting material.

Significance to Bio-medical Research and the Program of the Institute: These results further demonstrate that the proteins of chromatin restrict template activity in a tissue-specific manner. They show that the information for specific assembly is also present in chromatin, so that it should be possible to assay for and purify those protein factors which control globin specific transcription. Finally, the use of chemical probes to produce open and covered DNA, makes it possible to relate the distribution of proteins on DNA to the activation of the globin gene for transcription, a valuable step in relating chromatin structure to function. All of these properties are clearly related to the molecular mechanism of cellular differentiation in eukaryotes, and provide one of the best defined systems presently available for study.

Proposed Course of Project: We propose to isolate and purify the components responsible for activation of globin gene transcription, using the techniques described above, and to study the molecular interactions involved.

Publications:

R. J. Clark and G. Felsenfeld: Chemical probes of chromatin structure.

Biochem., in press.

R. Axel, H. Cedar and G. Felsenfeld: Chromatin template activity and chromatin structure. Cold Spring Harbor Symposium on Quantitative Biology XXXVIII, in press.

Gary Felsenfeld and Howard Cedar: Chromatin structure in solution. Fogarty Center Symposium Conference on Regulation of Gene Expression in Eukaryotic Cells, in press.

Howard Cedar, Richard Axel and Gary Felsenfeld: Structure and function of the globin genes in chromatin. Fogarty Center Symposium Conference on Poly (ADP-Ribose), in press.

Serial No. NIAMDD-LMB-14
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Calorimetric Investigations of Cellular Systems and Biological Macromolecules

Previous Serial Number: NIAMDD-LMB-10, NIAMDD-LMB-11

Principal Investigator: Philip D. Ross

Other Investigators: (none)

Cooperating Units: Dr. James Hofrichter NIAMDD-LCP
Dr. Wm. A. Eaton, NIAMDD-LCP
Dr. Robert N. Goldberg, N.B.S.

Man Years:
Total: 2.0
Professional: 2.0

Project Description:

Objectives: The absorption and liberation of heat is an extremely general phenomena accompanying most physical and chemical change. In the work on cellular systems, the general aim of this research is to observe and interpret the thermokinetic behavior of cells growing in tissue culture under various sets of conditions. In the simpler biochemical systems studied, the aim of this work is to make direct energy measurements to obtain thermodynamic and, when possible, kinetic parameters associated with defined chemical events.

Methods: A differential microcalorimeter designed to accept the sterile, disposable plastic Petri dishes (35 x 10 mm) used in tissue culture has been constructed. A well thermostatted environment confers long term thermal stability on the instrument so that observations may be carried out over a period of several days and that changes in the rate of heat production of 1.5 microwatts occurring over a five hour period may be detected.

The differential scanning calorimeter described last year (LMB-11) has been extensively calibrated and tested.

Major Findings: 1.) Growth of Cells in Tissue Culture. Differences in the thermal output during growth of 3T3 cells and 3T3 cells transformed with SV40 virus have been found. The magnitude of these thermal effects are proportional to cell number and para-synchrony in thermal output has been observed.

2.) Kinetics and Thermodynamics of Hemoglobin S gelation (with Drs. Hofrichter and Eaton). Deoxyhemoglobin S in concentrated solutions aggregates and forms an ordered, birefringent phase-the so-called gel. It is this gel which distorts red cells upon deoxygenation, forming bizarre and sickled shapes. This system exhibits a temperature-induced phase transition which we are studying by a wide variety of physical techniques.

The enthalpy change for the transition has been determined by differential scanning calorimetry and the data obtained suggest the presence of slow kinetic effects. Using the calorimeter in the isothermal mode, it has been possible to observe the thermokinetics of gelation as a function of temperature.

It will be possible, from these results, to provide a detailed description and plausible molecular mechanism for the gelation of hemoglobin S.

Significance to NIAMDD Research: Knowledge of the basis of the events involved in the gelation of hemoglobin S will provide a sound framework with which to interpret the effects of theraputic reagents upon the sickling process.

Proposed Course: The thermokinetics of cellular growth will be studied while simultaneously monitoring a large number of biochemical changes in order to relate the heat production to various stages and events in the cell cycle. This work will provide the necessary basis for the interpretation of differences in thermal behavior already found for different mutants.

The study of the gelation of hemoglobin S will be extended to determine the thermodynamic and kinetic effects of other hemoglobin species, i.e. minor blood components and other mutant hemoglobins which may or may not enter into the ordered phase. These studies will be extended to the sickling process in erythrocytes.

Publications:

Ross, P. D., Fletcher, A. P., and Jamieson, G. A.: Microcalorimetric Study of Isolated Blood Platelets in the Presence of Thrombin and Other Aggregating Agents. Biochimica et. Biophysica Acta 313: 106, 1973.

Ross, P. D.: Microcalorimetry Techniques: Applications to Cellular Systems. Science 183: 227, 1974.

Ross, P. D. and Shapiro, J. T.: Heat of Interaction of DNA with Polylysine, Spermine and Mg^{++}. Biopolymers 13: 415, 1974.

Ross, P. D. and Scruggs, R. L.: Heat of Poly (rU) Helix Formation Induced by Spermine. Biopolymers 13: 417, 1974.

Serial No. NIAMDD-LMB-15
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Enzymes from Mammalian Tissues which are Involved in the Repair or Replication of DNA

Previous Serial Number: NIAMDD-LMB-14

Principal Investigator: Steven B. Zimmerman

Other Investigators: Cora Boswell
Patricia McKinley

Man Years:
Total: 1 1/4
Professional: 1/2
Other: 3/4

Project Description:

Objectives: To isolate and characterize several enzymes from mammalian sources which act on DNA, including polynucleotide kinase and polynucleotide ligase, in order to characterize them generally and in particular to test their interaction with DNA in various regions and types of chromatin.

Methods: Standard techniques of biochemistry.

Major Findings: A DNA ligase has been purified ca. 7000-fold from rat liver. The initial step of the purification is the isolation of nuclei; nuclear extracts are then chromatographed on phosphocellulose followed by DNA-cellulose. The ligase seals single-strand nicks in DNA with any of the four usual bases on either the 3'- or 5'-sides. It requires ATP and a divalent cation (Mg^{++} or Mn^{++}) for activity. At low Mg^{++} concentrations the activity is greatly stimulated by a variety of monovalent cations. Relatively small excesses of either monovalent or divalent cation above the amounts which give maximal activity lead to inhibition of activity. Poly (G) and poly (I) inhibit ligase activity; several other polyribonucleotides are not inhibitory. Low concentrations of inorganic pyorphosphate are inhibitory. The molecular weight of the ligase is estimated from gel filtration to be ca. 10×10^4 daltons.

The activity of the ligase on polylysine complexes with DNA has been examined to aid in interpreting experiments planned to test ligase activity on chromatin. The ligase has the same maximal velocity on uncovered regions of DNA-polylysine complexes as it does on DNA. It has little or no activity on "nicks" covered by polylysine.

The "polynucleotide kinase" from rat liver nuclei has been purified about 400-fold relative to liver extracts.

Significance to Bio-Medical Research and the Program of the Institute: The ligase and kinase, as well as other enzymes, are thought to function in repair and replication of DNA. Damaged regions in DNA have been implicated in a variety of disease states as well as in aging and differentiation. A large proportion of the DNA is covered with proteins in chromatin and the present project is aimed at assessing the ability of these enzymes to work on such a substrate. It is possible the enzymes may also serve as a probe of chromatin structure, itself a controversial subject.

Proposed Course: To extend the model studies with polylysine-DNA complexes to the kinase and to test both kinase and ligase capability on chromatin; to examine other subcellular fractions for distinguishable ligases; to characterize the kinase.

Serial No. NIAMDD-LMB-16
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Aggregation of Polynucleotides at Acid pH.

Previous Serial Number: NIAMDD-LMB-15

Principal Investigator: Steven B. Zimmerman

Other Investigator: (none)

Man Years:

Total:	1/4
Professional:	1/4
Other:	0

Project Description:

Objectives: To describe and better understand the mechanisms involved in the aggregation of polynucleotides at acid pH.

Methods: Standard techniques of biochemistry and biophysics.

Major Findings: Polynucleotides precipitate at well defined pH values. These pH values are dependent upon both the base composition and the ionic strength of the medium. At low ionic strength, all the polymeric components in solution tend to precipitate together. The pH value at which such co-precipitation occurs can be predicted based upon a simple model which requires charge neutralization between the bases and phosphate residues for insolubility, i.e., precipitation occurs at the net isoelectric point of the solution. Spectrophotometric pH values for some common nucleosides were determined for use in calculating the isoelectric points. At higher ionic strengths, there were instances of fractional precipitation. Also at higher ionic strengths, several homopolymers (poly A, poly G, and to a lesser extent, poly U) had a second zone of insolubility near neutral pH values.

Significance to Bio-Medical Research and the Program of this Institute: Interactions of polynucleotides with themselves and with other molecules are basic factors in the successful functioning of a cell. The current studies are one facet of such interactions and may extend understanding to related phenomena.

Proposed Course: The publication listed below marks the completion of this project.

Publication: Zimmerman, S. B.: Precipitation of Polynucleotides from Acidic Solution. Effects of Base Composition and Ionic Strength. Biochemistry 12: 2916-2925, 1973.

Serial No. NIAMDD-LMB-17
1. Laboratory of Molecular Biology
2. Physical Chemistry
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: X-ray Diffraction Studies on Polyguanylic Acid

Previous Serial Number: NIAMDD-LMB-13

Principal Investigator: Steven B. Zimmerman

Other Investigator: David R. Davies

Man Years:
Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Objectives: To obtain structural information on poly G through x-ray fiber diffraction techniques.

Methods: Standard techniques of biochemistry and x-ray diffraction.

Major Findings: Model building studies taken in conjunction with our previous diffraction results have strongly indicated that both poly G and poly I have the same four-stranded structure.

Significance to Bio-Medical Research and the Program of the Institute: Both normal and diseased states of tissues are controlled to an impressive extent by polynucleotides. Structural studies on polynucleotides may offer new insights into the functioning of these molecules. The current work constitutes the first relatively strong evidence for the existence of new type of secondary structure, namely a four-stranded structure.

Proposed Course: The results of this project are being prepared for publication.

of ribosomes on the interaction has been carried out by equilibrium dialysis and by an improved filter-binding assay. GDP binds tighter than GTP by an order of magnitude (K_d = $4x10^{-7}$ and $4x10^{-6}$ respectively). Ribosomes inhibit the binding of GDP but not GTP. One possible interpretation of the data is that the guanosine nucleotides could modulate protein synthesis by affecting the binding of EF-2 to ribosomes and its release therefrom.

A project has been initiated to study the cell-free synthesis of the placental polypeptide hormones, HPL and HCG. This work will be done in collaboration with Dr. Saul Rosen and Bruce Weintraub, NIAMDD, CE.

Significance to NIAMDD Research: The enzymatic mechanisms in protein synthesis in mammals and the metabolic control of these processes is of major interest to biology and biochemistry. Our emphasis so far is on enzymatic mechanisms. Hopefully, understanding of control will follow.

Proposed Course: We will continue our efforts to identify the unknown basic amino acid at the site of the inactivation of EF-2 by diphtheria toxin and NAD^+ and to determine what role, if any, it plays in the process of translocation.

We will attempt to synthesize the placental polypeptide hormones in cell free systems, to isolate and purify the specific messenger RNAs and to look for control mechanisms at the levels of translation and transcription.

Publications:

Robinson, Elizabeth A., Henriksen, Ole and Maxwell, Elizabeth S.: The Site of ADP-Ribosylation of Elongation Factor 2, in Poly (ADP-ribose): An International Symposium, U.S. Govt. Printing Office (1974) in press.

Robinson, Elizabeth A., Henriksen, Ole and Maxwell, Elizabeth S.: Elongation Factor 2: Amino Acid Sequence at the site of ADP ribosylation. J. Biol. Chem. (1974) in press.

Serial No. NIAMDD-LMB-18
1. Laboratory of Molecular Biology
2. Theoretical Molecular Biology
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Energy conversion in biology, especially muscle contractio.

Previous Serial Number: None

Principal Investigator: Dr. T. L. Hill (12/12)

Other Investigators: Dr. Y. Chen (12/12); Dr. A. Ben-Naim (12/12)

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives: To provide the necessary general formalism for the theoretical treatment of muscle contraction, active transport, and related topics in biology, together with the application of these generalities to special cases suggested by experiment.

Major Findings: A detailed formalism has been developed that is required for the proper formulation and application of any model of the "sliding filament" type. Topics that have been worked out include steady and transient mechanics, work produced, ATP flux, thermodynamic efficiency, multiple actin sites, the near-equilibrium special case, stochastics, noise, and various special cases. It is planned in the next year to compare the predictions of particular models with experiment, and to extend the formalism to active transport.

Significance to Biomedical Research and the Program of the Institute: An understanding of the operation, at the molecular level, of a normal striated muscle is prerequisite to a rational attack on various muscular and neuromuscular abnormalities. A similar comment can be made about the active transport of ions and molecules across cell membranes.

Publications:

Hill, Terrell L.: Theoretical formalism for the sliding filament model of contraction of striated muscle (Part I). Prog. Biophys. Mol. Biol. 28, (in press) 1974.

Hill, Terrell L.: Theory of muscular contraction extended to groups of actin sites. Proc. Nat. Acad. Sci. 70, 2732, 1973.

Chen, Yi-der and Hill, Terrell L.: Analysis of a simple prototypal muscle model near to and far from equilibrium. Proc. Nat. Acad. Sci. 71 (in press) 1974.

Hill, Terrell L. and Chen, Yi-der: Further analysis of a simple prototypal muscle model near to and far from equilibrium. Proc. Nat. Acad. Sci. (in press) 1974.

Chen, Yi-der: Fluctuations and noise in kinetic systems. II. Open ensembles at equilibrium and steady state. J. Chem. Phys. 59, 5810, 1973.

Ben-Naim, A.: Generalized molecular distribution functions. J. Chem. Phys. 59, 6535, 1973.

Serial No. NIAMDD-LMB-19
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol. Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Statistical thermodynamics of polynucleotide - complementary monomer interactions

Previous Serial Number: NIAMDD-LMB-16

Principal Investigator: Dr. T. L. Hill (O)

Other Investigators: Dr. Y. Chen (O), Dr. N. Saito (O)

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives: (a) To clarify the thermodynamic procedures which should be used in comparing optical, calorimetric, and binding measurements on these systems; and (b) to attempt to interpret these experiments in terms of a statistical mechanical model.

Major Findings: Work on this project has been temporarily discontinued but will be resumed at a later date.

Serial No. NIAMDD-LMB-20
1. Laboratory of Molecular Biology
2. Section on Theoretical Mol. Biol.
3. Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Ion transport across the nerve axon membrane

Previous Serial Number: NIAMDD-LMB-17

Principal Investigator: Dr. Terrell L. Hill (O)

Other Investigators: Dr. Y. Chen (O)

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives: To attempt to interpret experimental kinetic, steady-state, and noise data on K^+ (especially) and Na^+ transport across the nerve axon membrane in terms of a model in which the channels are protein complexes undergoing voltage-dependent conformation changes.

Major Findings: Work on this project has been temporarily discontinued but will be resumed at a later date.

Annual Report of the

Mathematical Research Branch

National Institute of Arthritis, Metabolism, and Digestive Diseases

The computer-assisted system, reported last year, for recording the present illness section of the medical history has been revised and [illegible]eted. The complete description is in press (Assisted recording of the medical history (ARM): A method of recording medical history during the [illegible]. Comput. in Biol. and Med., in press). (Dr. S. Goldstein)

A mathematical model for ketoacidosis in diabetes is being developed. Recently questions have been raised regarding insulin resistance or the reduction of effectiveness of insulin by acidity and ketone bodies. Initial findings from the model suggest that insulin resistance is a real effect but quantitatively less important than usually assumed. (Dr. S. Goldstein)

A numerical and theoretical study of the interaction between geometric and electrical attributes of neurons has been made. It has been shown that in tapered (or flared) core conductors the propagation velocity changes in proportion to the change in characteristic length. A simple approximation between degree of taper (or flare) and propagation velocity has been derived. This work has been submitted for publication. (Dr. W. Rall and Dr. S. Goldstein)

Extensive research has been pursued on transient responses to current injected at one branch of a dendritic neuron. The response function (transfer function) has been derived for injections of current at a given site with observation at a distinct site. Superposition then gives the response for observation at a given site with arbitrary injection. This work has been submitted for publication. (Dr. W. Rall and Dr. J. Rinzel, DCRT)

Computations have been performed in order to achieve a better understanding of electrical recordings from large groups of synchronously active cells. In collaboration with Dr. W. Rall, the effects of spatial distribution, type of activity, degree of synchrony and inhomogeneities in the conducting medium have been explored. (Dr. M. Klee)

Electrical recordings from physiological preparations are analyzed by methods which assume the presence or absence of three dimensional effects. It has been shown theoretically that a concomitant of such effects is a "phase resonance" in potentials produced by sinusoidal stimulation. In collaboration with Dr. D. Carpenter and D. Livergood experiments are being designed to search for such resonance in Aplysia neurons. (Dr. M. Klee)

A formulation describing facilitated diffusion of oxygen in the presence of hemoglobin or myoglobin has been developed. Piecewise linear approximation of the nonlinear chemical kinetics of binding of oxygen yields a piecewise linear analytical solution with continuity of flux at each change over point. The method is applicable when the diffusion region is partitioned by one or more membranes impermeable to hemoglobin or myoglobin. It is thus possible

to assess the role in muscle of facilitation classically studied on millipore membranes. (Dr. J. Gonzalez-Fernandez and Mrs. S. Atta)

The general theoretical analysis of the renal counterflow system has continued. Two papers on the details of the "central core" model appeared in 1973 (Biophys. J. 13:512-545, Ibid 13:546-567). A general thermodynamic analysis of free energy balance has been carried out (Math. Biosci., in press). This analysis provides the basis for computation of power utilization in various transport processes. (Dr. J. Stephenson, NHLI, Associate Member, MRB)

A major effort has been directed toward numerical solution of the differential equations describing the steady-state behavior of the kidney. Some of the earlier work on "shooting" methods has now been published (Proceedings of 1973 Summer Computer Simulation Conference, Vol. 2, pp. 806-810). During the past year (in collaboration with Prof. R. Tewarson, SUNY, Stony Brook) some highly efficient Newton-type methods have been developed and used to investigate the behavior of the counterflow system. The computational results have shown that the central core model gives a very useful approximate description of the behavior of the coupled nephrovascular counterflow system. The numerical solutions have also set some order of magnitude bounds on permeability requirements for concentration in different models. These bounds are in agreement with experimentally measured permeabilities. The calculations have shown among other things that it is quite possible NaCl transport out of thin ascending limb of Henle is entirely passive, being induced by urea entry into the inner medulla from collecting duct. By combining the numerical solutions with the thermodynamic analysis it has been possible to compute the power utilized for solute concentration versus the power dissipated in transmembrane transport of solutes and water. A preliminary report on this work is in press (Proc. Nat. Acad. Sci., USA, May issue). (Dr. J. Stephenson and Mr. R. Mejia, NHLI, Associate Members, MRB)

The results reported last year for maximum increasing matrices have been extended to time dependent matrices. Let $\dot{x}=Ax$, where A is integrable with nonnegative off diagonal entries and nonpositive row sums. If Φ is the fundamental matrix of the system, then Φ is a maximum decreasing matrix. This includes the case of constant A giving $\Phi = \exp(At)$ and all of the previous inferences carry over to the time variable case. (Dr. J. Hearon)

The existence of solutions of the matrix equation $XA-BX=C$ is well studied. An important question in control and observer theory of linear systems is the existence of an invertible solution, a question studied thus far only when rank C is one. Sufficient conditions have been found for the general case and necessary conditions when the spectra of A and B are disjoint. (Dr. J. Hearon)

Studies of 3-dimensional reconstruction have continued. An algorithm for recognizing and locating artifacts in a reconstruction has been devised and demonstrated. These results were published in proceedings of the 1st International Joint Conference on Pattern Recognition. The relation between certain versions of the reconstruction equations and partial differential equations has been explored with the possibility well known techniques in differential equations can be brought to bear on the reconstruction problem. (Dr. R. Gordon)

Serial No. NIAMDD, ODIR, MRB-1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mathematics of kinetics and reaction-transport systems

Previous Serial Number: SAME

Principal Investigator: John Z. Hearon

Man Years:

Total:	1.3
Professional:	1
Others:	.3

Project Description:

Objectives: The objectives of this research remain substantially as stated in previous reports.

Major Findings: In addition to consultative and collaborative activity, efforts during the past year were concerned with the following work:

Maximum Increasing Matrices.

The work reported last year for constant matrices has now been extended to time dependent matrices. If $\dot{x}=A(t)x$ where $A(t)$ is any integrable matrix function of t then $x(t)=\Phi(t,t_0)x(t_0)$ where $\Phi(t,t_0)$ is the fundamental matrix of the system. It has now been shown that if for all $t > t_0$

$$a_{ij} \geq 0 \quad \text{for } i \neq j$$
$$\sum_j a_{ij} \leq 0 \quad \text{for all } i \qquad (1)$$

Then $\Phi(t,t_0)$ is a maximum decreasing matrix. That is for every vector v

$$\max(\Phi v) \leq \max(v)$$

where

$$\max(v) = \max_i v_i$$

Thus in any time dependent linear system such that the matrix obeys (1) we have the result that at no time can any entry in the solution vector exceed the maximum entry in the initial vector. It also results from the method of proof that $\|\Phi\| \leq 1$ for all $t \geq t_0$ where $\|\Phi\|$ is the row-sum norm of the matrix Φ which is consistent with the vector norm $\|v\| = \max|v_i|$. Thus time dependent matrices with property (1) yield fundamental matrices with norm never greater than one.

Nonsingular Solution for an Operator Equation.

An important problem in system theory is the following.

Let the state vector of the system

$$\dot{x} = Ax$$

be the input to the system

$$\dot{z} = Bz + Cx$$

Then $z = Tx$ if and only if T is a solution of

$$TA - BT = C \qquad (2)$$

In this case the state vector x can be recovered from the response vector z of the second system if T has an inverse. The existence of an invertible solution of (2) is thus of great interest and has been studied only for the case that rank C = 1 and the spectra of A and B are disjoint. We have the following results:

(1) a very direct proof in the rank one case that the controllability of [B,C] and observability of [A*,C] are sufficient with no restriction on the spectra.

(2) a proof of the necessity of the above conditions in the general case when the spectra of A and B are disjoint

(3) a set of sufficient conditions in the general case showing why the rank one case is so special and has been so readily solved in the past.

Completely Unobservable Linear System.

In continued study of lumping in linear systems we have constructed a completely unobservable system. A system is observable if the output on an interval (0,t) suffices to determine the initial state. It is completely unobservable if there is a linear variety such that no two initial states in the variety can be distinguished. We have the result:

Let $\dot{x} = Ax$

where

$$A = \begin{pmatrix} A_1 & A_{12} \\ A_{21} & A_2 \end{pmatrix}$$

and A_1 is k-square. Let $x^*(0) = [\xi^*, \eta^*]$ where ξ is a k-vector. Then $x_1, x_2, \ldots x_k$ are invariant under initial vectors such that the differ by vector $\Delta^* = [0, q]$ where q is in some invariant subspace of A_2 and the null space of A_{12}. In short under these physically achievable circumstances if the output consists of knowledge of the first k entries of the state vector there are an infinite number of initial vectors giving the same output.

Publications:

Hearon, J. Z.: Distribution of conserved species in diffusion-reaction systems. Bull. Math. Biology 35: 59-67, 1973.

Hearon, J. Z.: A note on open linear systems. Bull. Math. Biology 35, 1974 (In press).

Serial No. NIAMDD, ODIR, MRB-2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mathematical formulation and analysis of problems relevant to experimental neurophysiology

Previous Serial Number: SAME

Principal Investigator: Wilfrid Rall

Other Investigators: Steven Goldstein
John Rinzel
Maurice Klee

Man Years:

Total:	1.3
Professional:	1.0
Others:	.3

Project Description:

Over the past several years, this project has been concerned with constructing, developing and testing a group of interrelated mathematical models. This group of models provides a theory that can account for many different sequences of events in a dendritic neuron, and in certain populations of such neurons. Computational experiments performed with these models provide theoretical predictions that have been compared with the results of neurophysiological experiments and neuroanatomical observations. Also, insights have been obtained, which have led to new hypotheses, new interpretations, and to the design of new experiments.

1. (With Dr. Goldstein). Computational exploration of action potential propagation in regions of changing geometry (core conductor taper, branching and step changes in diameter) has been mentioned previously and is now being submitted for publication. Additional theoretical results for tapered core conductors are being prepared for publication.

2. (With Dr. Rinzel, Laboratory of Applied Studies, DCRT). Our first paper (Branch input resistance and steady attenuation for input to one branch of a dendritic neuron model) was published in the Biophysical Journal in July 1973, and our second paper (Transient response in a dendritic neuron model for current injected at one branch) is now being submitted for

publication. The implications relating to dendritic spines have been presented at several symposia and will be prepared for publication during the next fiscal year.

3. (With Dr. Klee). Presentation and analysis of the characteristics of electric fields generated by activity in a single neuron and in open and closed cortical arrangements of neuron populations. Extensive computations have been begun with special attention to the preparation of illustrations designed to be useful to experimental neurophysiologists. This effort will present both old and new theoretical results in a comprehensive context, probably as a set of three related theoretical papers.

4. Core conductor theory and cable properties of neurons. Literature research, organization of material and considerable writing effort have been devoted to preparation of a chapter for the new Handbook of the Nervous System, Volume I, Cellular Biology of Neurons, that is now in preparation for The American Physiological Society.

Advisory Councils:

Central Council of IBRO (International Brain Research Organization, affiliated with UNESCO); 1968-1973.

Committee on Brain Sciences, National Research Council; 1968-1973.

USA National Committee for IBRO, National Research Council; 1972-1976.

Publication:

Rall, W. and Rinzel, J.: Branch input resistance and steady attenuation for input to one branch of a dendritic neuron model. Biophys. J. 13: 648-688, 1973.

Serial No. NIAMDD, ODIR, MRB-3
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mathematical description of the transport-chemical reaction kinetics of substances in the blood-capillary-tissue complex

Previous Serial Number: SAME

Principal Investigator: Jose M. Gonzalez-Fernandez

Other Investigators: Susie E. Atta

Man Years:

Total:	2.3
Professional:	2
Others:	.3

Project Description:

Objectives: The broad objectives of this research remain substantially the same as the ones stated in the previous reports.

Major Findings:

Nonsteady state:

The work on nonsteady state as outlined in the previous report is being written up for publication.

Facilitated oxygen diffusion:

A formulation that describes the facilitated diffusion of oxygen in the presence of hemoglobin or myoglobin has been developed. By approximating the nonlinear chemical kinetics in a piecewise linear manner, a piecewise linear analytic solution was obtained, with matching flux conditions at every changeover point. This set of matching conditions is expressed as a nonlinear algebraic problem that was solved numerically by the Newton method. This method lends itself to the study of the facilitated diffusion when membranes not permeable to hemoglobin or myglobin are in the diffusion path; this situation exists for the diffusion of oxygen in muscle. So this opens the possibility of assessing how much of the experimental facilitation on millipore membranes may exist in the muscle itself.

Numerical analysis:

The algorithm to improve the rate of convergence for the point successive iterative process as described in the previous report has been written and the manuscript will be submitted for publication.

Publications:

Gonzalez-Fernandez, J. M. and Atta, S. E.: Maximal substrate transport in capillary networks. Microvascular Research 5(2): 180-198, 1973.

Serial No. NIAMDD, ODIR, MRB-4
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Applications of electric field theory to electrophysiology

Previous Serial Number: SAME

Principal Investigator: Maurice Klee

Man Years:

Total:	1.0
Professional:	1.0
Others:	0

Project Description:

Objectives: To obtain an understanding of the electrical behavior of nerve and muscle in terms of three dimensional electric field theory.

Methods: Numerical and analytic techniques are used to calculate the electric fields within and surrounding both single cells and groups of cells.

Major Findings and Significance: During the past year, this project has emphasized three areas: (1) the electric fields generated by groups of synchronously active cells, (2) experimental methods for identifying three dimensional effects in electrophysiological preparations, and (3) the role of cell shape during extracellular stimulation.

In collaboration with Dr. Wilfrid Rall, a series of mathematical calculations have been performed in order to obtain a better biophysical understanding of the experimental recordings from large groups of synchronously active cells. The parameters explored include: the spatial distribution of the active cells, the type of electrical activity exhibited by the cells, e.g. synaptic versus action potential activity, the degree of temporal synchrony among the cells, and the effects of inhomogeneities in the conductivity of the medium in which the cells are imbedded. Each of these parameters was found to significantly alter the waveform recorded from a group of active cells.

The existence of three dimensional effects in electrophysiological preparations depends upon the electrical parameters and geometry of the system, factors which in general are only partially known. The appropriate analysis of experimental data requires a decision on whether or not three

dimensional effects are to be considered. Theoretically, it has been shown that a concomitant of the existence of three dimensional effects is the existence of a "phase resonance" in the electric potentials produced by sinusoidal intracellular stimulation. In collaboration with Drs. David Carpenter and David Livengood of the Armed Forces Radiobiology Institute a series of experiments are being designed to search for such a resonance in the somas of Aplysia neurons. Theoretical calculations indicate that the parameters of this preparation should be just critical for three dimensional effects.

Biological cells exhibit a wide variety and great intricacy of cell shapes. In a theoretical study using oblate and prolate spheroids, the effect of such shapes upon the response of cells to extracellular stimulation has been examined. For equivalent stimulating conditions, oblate shaped cells were found to generate significantly larger transmembrane potentials than either spherical or prolate shaped cells. For a changing direction of stimulation, a cell's shape was shown to impart to the cell a directional sensitivity to stimulation, i.e., the cell responded more strongly to some directions of stimulation than to others. Although the magnitude of the response changed with changes in stimulating direction, the location of the maximal response remained relatively constant. This effect provides a convenient mechanism available to the nervous system for electrically summing the effects of spatially distributed sources.

Serial No. NIAMDD, ODIR, MRB-5
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Theoretical effects of geometry on the electrophysiology of neurons

Previous Serial Number: MRB-6

Principal Investigator: Steven Goldstein

Man Years:

Total:	.7
Professional:	.65
Others:	.05

Project Description:

Objectives: To determine theoretically how the geometric properties of a neuron interacts with the electrical properties of the neural membrane. (In collaboration with Dr. W. Rall, MRB, NIAMDD)

Methods: Further numerical calculations were performed with the *Veg* model.

Results Obtained: Calculations were performed in core conductors where the radius increased (or decreased) with distance. It was found for a particular class of such flaring (or tapering) core conductors that velocity increased or decreased in exact proportion to the changing length constant, λ. Furthermore, a simple approximation was found for the relation between the amount of flare (or taper) and the velocity.

The shape and velocity of action potentials near regions of step change in diameter has been defined.

A paper entitled "Changes of Action Potential Shape and Velocity for Changing Core Conductor Geometry" has been submitted for publication.

Serial No. NIAMDD, ODIR, MRB-5.1
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Automated recording of the medical history

Previous Serial Number: MRB-6.1

Principal Investigator: Steven Goldstein

Man Years:

Total:	.15
Professional:	.1
Others:	.05

Project Description:

Objectives: Previously, it was determined that it is possible to record the relevant medical history during the physician-patient interview. After this preliminary study it was decided to develop the system further in a clinical setting.

Methods: Major Changes - The only major change in the system was a method of editing the history once it is recorded. This was felt to be necessary to handle the situation that arises when a patient corrects information that he has given earlier in the interview.

Proposed Course: Although no major interest for the system has been generated in the Washington, D.C. area, it is hoped that the publication cited below will generate interest elsewhere.

Publication:

Goldstein, S.: Assisted recording of the medical history (ARM): A method of recording the medical history during the interview. Comput. in Biol. and Med. (In press).

Serial No. NIAMDD, ODIR, MRB-5.2
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Theoretical aspects of diabetic ketoacidosis

Previous Serial Number: NONE

Principal Investigator: Steven Goldstein

Man Years:

Total:	.25
Professional:	.25
Others:	0

Project Description:

Objectives: The treatment of diabetic acidosis has been empirically developed. Recently, traditional treatment has been questioned in the literature. The controversy basically revolves about the question of whether "insulin resistance" exists, i.e., whether or not pH or ketoacids affect the blood sugar lowering effect of insulin.

Methods: In order to answer this question, it was decided to develop a mathematical model of ketoacidosis based on data obtained from the treatment of patients. Arrangements have been made to obtain relevant clinical data from D.C. General Hospital, Washington Hospital Center and the V.A. Hospital of Washington, D.C.

In addition an initial model has been constructed from available data in the literature.

Major Findings: Although no data has yet to be collected, the initial model suggests that although insulin resistance is a real phenomenon, it is of less importance in the management of ketoacidosis than previously thought.

Serial No. NIAMDD, ODIR, MRB-6
1. ODIR
2. Mathematical Research Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Three dimensional reconstruction from projections

Previous Serial Number: MRB-7.1

Principal Investigator: Richard Gordon

Other Investigators: Marcello Barbieri (Guest Worker) and R. L. Kashyap

Cooperating Units: Institute of Histology and Embryology, University of Bologna, Italy; School of Electrical Engineering, Purdue University, Lafayette, Indiana

Man Years:

Total:	0.55
Professional:	0.55
Others:	0

Project Description:

Objectives: The general problem is to ascertain the internal structure of an object from its projections, such as x-rays of a patient, taken from a number of angles. The particular objectives are: 1) to develop the theory of reconstruction from finite numbers of projections; 2) to obtain the three dimensional structure of ribosomes using this theory; 3) to apply the theory to medical radiology.

Major Findings:

1) To the molecular biologist or the radiologist, a question more important than the resolution of a reconstruction from projections is the reality of the features it contains. An algorithm for locating artifacts in reconstructions was devised and demonstrated. Results of this work were presented at a conference on pattern recognition and published.

2) The reconstruction equations were reformulated as partial differential equations. Since the reconstruction equations are highly underdetermined, one may seek the solution minimizing some prechosen cost function. The spatially continuous limit of one such cost function minimizing local discontinuities lead to variational formulations which may be converted to

partial differential equations. Another cost function attempts to match the expected autocorrelation for a given class of objects and may lead to much improved reconstructions. The reconstruction problem was also expressed as a solution to a diffusion equation. Thus, reconstruction can now be analyzed by standard methods of numerical analysis. (With R. L. Kashyap)

3) A tutorial article on ART algorithms (Algebraic Reconstruction Techniques) was prepared, which led to a rigorous formulation of the geometry of reconstruction, and to recommendations for more efficient computing.

4) Work was begun on creating computer programs for preparing densitometer scans of ribosomes for 3-dimensional reconstruction. (With M. Barbieri)

5) A preliminary algorithm was devised which could be implemented as an analog optical device (ARTIST - ART Intended for a Storage Tube). Such a device may be capable of obtaining three dimensional resolutions ten times finer than the EMI-Scanner, in a shorter time.

Significance to Biomedical Research:

Three dimensional reconstruction has become an important diagnostic technique in medical radiology. The work outlined above should lead to higher spatial resolution, lower x-ray dosages to patients and more reliable medical interpretation of reconstructions than current methods allow.

The structure and functioning of the ribosome is one of the outstanding unsolved problems of molecular biology. The work begun on its three dimensional structure may make a major contribution to this field.

Proposed Course:

1) Interactive image processing computer programs will be completed for handling densitometer scans of ribosome microcrystals.

2) Preliminary reconstructions of ribosomes will be made.

3) An accurate ARTIST algorithm will be sought, and if found, a prototype instrument may be built.

4) Data will be sought from the EMI-Scanner to attempt to obtain improved reconstructions.

Publications:

Gordon, R. and Herman, G. T.: Three dimensional reconstruction from projections: a review of algorithms. Int. Rev. Cytol. 38: 111-154, 1974.

Gordon, R.: Artifacts in reconstructions made from a few projections. Proc. 1st Int. Joint Conf. on Pattern Recognition, 30 October to 1 November, 1973, pp. 275-285.

Gordon, R.: A tutorial on ART (Algebraic Reconstruction Techniques). IEEE Trans. Nucl. Sci. (In press)

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Computer simulation of the change of shape of the newt neural plate during early embryogenesis

Previous Serial Number: MRB-7.3

Principal Investigator: Richard Gordon

Other Investigators: Antone G. Jacobson

Cooperating Units: Zoology Department, University of Texas, Austin

Man Years:

Total:	0.5
Professional:	0.5
Others:	0

Project Description:

Objectives: To explain how the pattern of cell behavior transforms the vertebrate neural plate (the embryonic brain) from a hemispherical to a keyhole shaped sheet of cells.

Major Findings:

1) Modifications of the numerical methods in the computer program SHRINK (Simulated Hydrodynamic Reshaping into Neural Keyhole) led to a successful simulation. The transformation of the newt neural plate from a disc to a keyhole shape was accomplished using two forces: nonuniform shrinkage due to elongation of cells perpendicular to the plate, and shoving by the growing notochord beneath the plate.

2) A difference was found between the shapes of the excised and intact neural plate, which may be attributed to a third force: stretching by the epidermis around the back of the intact embryo.

3) The general problem of morphogenesis was formulated in terms of continuum mechanics. Such "morphodynamics" is distinguished by fluid elements (cells) which grow and exert active forces on neighboring elements.

Significance to Biomedical Research: The computer simulation and mathematics which were developed should be widely applicable to problems of organogenesis and form in general. These methods allow one to span the gap between cell behavior and the structure of organisms.

Proposed Course:

1) Computer simulations imitating experimental surgery on the newt embryos will be undertaken.

2) The spatial distribution of the height programs of the cells will be measured directly from three dimensional reconstructions of sectioned embryos.

3) A manuscript is being prepared for submission to Developmental Biology.

ANNUAL REPORT OF CLINICAL INVESTIGATIONS, NIAMDD

July 1, 1973 to June 30, 1974

Phillip Gorden, M.D., Clinical Director

The major change in NIAMDD Clinical Investigations has been the departure of Dr. Robert S. Gordon to become Director of the Clinical Center. Dr. Gordon maintains his link to NIAMDD, however, by remaining Chief of the Digestive Diseases Branch. Dr. Phillip Gorden became Acting Clinical Director in January of 1974, and in April was appointed officially as Clinical Director. Dr. Gerald Aurbach has replaced Dr. Robert Gordon as Chief of the Metabolic Diseases Branch; and Dr. Stephen Marx returned to the Branch as a Senior Investigator, after an absence of more than a year at the Massachusetts General Hospital. Otherwise, the senior staff has remained constant and without other major fluxes.

In other areas of reorganization, the beds for the Metabolic Diseases Branch have been transferred from 9-West to 8-West in order to consolidate patients with endocrine-metabolic diseases. This was done in response to the new program in the endocrine-metabolic area where Clinical Associates from NICHHD, NHLI and NIAMDD rotate among the different Institutes. This program, headed by Dr. Bruce Weintraub, increases the scope of both training and opportunity for collaborative research and has the enthusiastic support of both the Clinical Associates and senior staff.

For the period April 1, 1973 to March 31, 1974, the statistics with regard to patient admissions and outpatient visits changed very little from the previous year. There were 548 inpatient admissions for a total of 13,662 hospital days, yielding an average length of stay of 24 days. The average bed occupancy was 59%. There were altogether 2,894 outpatient visits to the several clinics operated by NIAMDD.

Data on the inpatient admissions have been sorted by computer and give rise to the following tables, which may be informative:

Admissions by Nursing Unit	
9-East	235
9-West	154
8-West	159
Total	548

Admissions by Branch		Assigned Beds	Admissions per Bed	Clinical Associates on Duty
ARB	207	15	13.8	7
CEB	115	14	8.2	5
DDB	97	14	6.9	4
PMB	64	6	10.7	2
MDB	51	6	8.5	2
CHB	14	7	2.0	2
Total	548	62	8.9	22

Admissions by Admitting Diagnosis

Acromegaly	18
Cystic fibrosis	63
Diabetes	12
Gilbert's syndrome	11
Hypercalcemia/hyperparathyroidism	34
Interstitial pulmonary fibrosis	22
Normal volunteers	37
Obesity	11
Rheumatoid arthritis	40
Systemic lupus erythematosus	115
Sprue	12
Thyroid carcinoma	13
Others	160
Total	548

During FY 1974 there were 22 Clinical Associates on duty with NIAMDD. Other temporary professional and nonprofessional staff included five Visiting Scientists and 27 guest workers, representing seven foreign countries in addition to the United States.

Dr. Henry Metzger was asked to head a new section, Section on Chemical Immunology. This unit now acts in parallel with sections on Human Biochemical Genetics (Dr. Neufeld) and on Connective Tissue Disease (Dr. Decker).

Dr. John van Boxel, Visiting Scientist, joined the branch at the beginning of the report period and has continued his work on aspects of lymphocyte physiology.

I. Rheumatoid Arthritis and Models

Work is in progress on the effect of cyclophosphamide on the rheumatoid synovia (Decker, Aptekar, Cattell (not NIH) and Hopps (BB)). Striking reductions in lymphocyte populations have been observed with decreasing inflammation. The nature of these lesional lymphocytes has been assessed with the application of a variety of immunological techniques to the cells recovered from digestates of synovial specimens; they appear to be largely of the thymic dependent or T cell class (van Boxel, Paget, Decker).

Aspirin, regularly used in large doses in the management of the disease, has been found to be the cause of occasionally observed serum enzyme changes, themselves caused by hepatocellular injury. In clinical studies progressively larger doses of aspirin have been found to produce such serum changes in 3 of 18 people with rheumatoid arthritis and 5 of 14 with systemic lupus erythematosus. In a retrospective analysis of 18 patients on our service with elevated transaminase values, 13 were found who developed the values due to aspirin administration; other causes were identified in 5 (Seaman, Plotz).

II. Systemic Lupus Erythematosus and Models

The results in 38 patients with lupus nephritis who were randomly assigned to cyclophosphamide, azathioprine or prednisone are now under analysis. The first patients were admitted just over 4 years ago and the last 1-1/2 years ago as of this writing (May 1974). The drugs have not conferred an extended advantage to date. There have been 8 deaths and 3 patients have had to go onto chronic extracorpeal dialysis; counting the latter as dead, our survival rates (by life table methods) are well in excess of those in the literature for this severe class of disease (Decker, Steinberg, Plotz, Klippel, Aptekar).

The presence and continued study of a large group of patients with the disease has permitted a number of related investigations of such problems as the incidence of bacterial infections (Decker, Staples, Gordon, Gerding), the nature of the central nervous system problems (Hadler, Gerwin (NINDS), Frank (NIAID), Whitaker (NINDS), Baker (NIMH), Decker)), an analysis of fibrin degradation products in the urine as an index of activity of nephritis (Decker, Steinberg, Gralnick (CC-CP), Marchesi (CC-CH), Aptekar)), and the modifications of immune status which may be observed (Decker, Steinberg, Aptekar, Waldmann (C:MET)). Tubuloreticular structures in the lymphocyte

cytoplasm have been found in the cord blood of two healthy infants born to mothers with systemic lupus (Decker, Grimley (NCI), Klippel)). The first in vivo identification of Libman-Sachs endocarditis as a cause of profound congestive heart failure made possible surgical correction; the patient is alive and well (Paget, Bulkley (NHLI), Grauer (NHLI), Seningen (CC-R)).

The finding that the anti-DNA antibodies in the circulation of patients with nephritis differ qualitatively from those free of renal disease may be of great importance in the near future (Steinberg, Gershwin, Gerber, Scher (not NIH)).

In the spontaneous mouse model of lupus increasing evidence fortifies the concept of a loss of suppressor function of certain classes of thymic dependent lymphocytes (Steinberg, Gerber, Gershwin, Gelfand (NIAID), Scher (NNMC), Gazdar (NCI), Chused (NIDR), Morse (NIAID)), a deficit which can in part be remedied by intermittent thymus grafting to syngeneic hosts (Steinberg, Gerber, Gershwin, Gelfand (NIAID)).

III. Relationships between Circulating Immunoglobulins and Cells

A major effort to understand the "transducing" effect of IgE on the surface of mast cells or basophiles is in progress. Receptors for the protein on a leukemic rat basophile line which can be grown in spinner cultures have been identified and characterized (Metzger, Kulczycki, Isersky-Carter, Carson, Buell (NCI)). Rat IgE antibodies have been isolated in pure form (Metzger, Isersky-Carter, Kulczycki). A new method for quantitating circulating IgE was then developed (Metzger, Carson, Bazin (not NIH)) and studies of IgE structure are underway (Metzger, Halper).

The effect of IgE and anti-IgE in various concentrations on the basophil surface has been characterized. Taken together these studies mean that a cell surface immunoglobulin receptor can, for the first time, be rigorously studied. It is on this surface that many of the critical steps of the immune response transpire; this is where the action is.

The surface of the lymphocyte, too, is under study and it has been shown that activated thymus dependent (T) lymphocytes can be identified by their rosette forming potential and by their ability to bind aggregated γ-globulin, a property hitherto associated only with bone marrow derived (B) lymphocytes (van Boxel, Rosenstreich (NIDR)). These cells are not capable of killing in an antibody-dependent lymphoid cell mediated cytotoxicity system (van Boxel). Reasons for believing IgD on cell surfaces to be synthesized by that same cell and to function as a receptor were developed (van Boxel, Buell (NCI)).

Several other studies of immunoglobulin structure were carried out or reported from work done in earlier years. Waldenstrom macroglobulins were found to include at least one J-chain per molecule (Metzger, Mihaesco (not NIH), Mihaesco (not NIH)).

IV. Biochemical Lesions in the Genetic Mucopolysaccharidoses and Mucolipidoses

Progress has been made with two enzymes, α-L-iduronidase and sulfoiduronate sulfatase, deficient in the Hurler and Hunter syndromes respectively. α-L-iduronidase has been successfully purified from human urine and two isozymes have been detected; one is incapable of incorporation in fibroblasts, a point of potentially key importance since successful therapeutic use of the enzyme will presumably require fibroblast capture.

An assay for sulfoiduronate sulfatase has been perfected which will permit its purification. It may also make possible the detection of female carriers of the x-linked Hunter syndrome (Neufeld, Hickman, Shapiro, Lim, Hall, Scott).

Serial No. NIAMDD-ARB-1c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Synovial Changes in Rheumatoid Arthritis Under Cyclophosphamide Therapy

Previous Serial Number: None

Principal Investigator: John L. Decker, M.D.

Other Investigators: Robert G. Aptekar, M.D., Hereward Cattell, M.D. and Hope Hopps.

Cooperating Units: George Washington University Hospital and Bureau of Biologics, (DIR).

Man Years:

Total:	3/4
Professional:	1/4
Other:	1/2

Project Description:

Objectives:

Cyclophosphamide has been shown to be of substantial benefit in the management of rheumatoid arthritis; among other pertinent observations, one study showed an extraordinary reduction in the number of bone erosions which appeared over an 8 month period as compared to a control group not receiving the drug. This study was designed to evaluate the effect of the drug on the synovial membrane in which the destructive pannus appears.

Methods Employed:

Selected patients with severe rheumatoid arthritis deteriorating on more conventional forms of therapy and in need of one or another of a variety of effective surgical procedures are admitted. The needed surgery is carried out with the synovial specimen receiving both histological and virological study. They are begun on cyclophosphamide and, after stabilization, are returned to their referring physicians for care under our supervision. Six months later, they are readmitted and a second surgical procedure permitting synovial sampling is carried out with the tissue studied as above.

Major Findings:

In the first six patients whose course has been completed, preliminary analysis shows a profound effect on the "immunological elements" of the synovial process and little or no change in the "granulomatous." It has not been possible to isolate or otherwise identify viral agents from the tissues either before or after "immune suppression."

Significance to Bio-Medical Research and the Program of the Institute:

The study suggests that immunological events in synovial tissue are the stimulus to granulomatous destruction and that an actively eroding granuloma is not distinguishable histologically from a more quiescent form. The theory of the viral etiology of rheumatoid was not substantiated.

Proposed Course:

A few more patients are "in course." Upon their completion, the histological material will be analyzed in detail in parallel with radiological change.

Honors and Awards: None

Publications:

1. Decker JL, Healey LA: When to use cytotoxic drugs in rheumatoid arthritis. Geriatrics 28:103-106, 1973.

2. Wilkes RM, Simsarian JP, Hopps HE, Roth H, Decker JL, Aptekar RG, Meyer HM: Virologic studies on rheumatoid arthritis. Arthritis Rheum 16: 446-454 (July-August) 1973.

Serial No. NIAMDD-ARB-2c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Retrospective Evaluation of Knee Synovectomy in Rheumatoid Arthritis

Previous Serial Number: ARB-2c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Robert G. Aptekar, Dr. Leonard T. Peterson, Orthopedic Consultant, George Washington University School of Medicine and Dr. David Fried (CC:Rehab)

Cooperating Units: George Washington University School of Medicine, Washington, D. C. and Clinical Center, Rehabilitation Department.

Man Years:

Total:	3/4
Professional:	1/4
Other:	1/2

Project Description:

Objectives:

To discover whether or not prophylactic synovectomy, as defined below, will alter the functional or structural prognosis in rheumatoid arthritis.

Methods Employed:

Patients with rheumatoid arthritis who have had at least six months of active inflammation in both of a pair of symmetrical joints, neither of which show radiological changes other than osteoporosis, have been admitted to the study. The disease must be sufficiently symmetrical to have all parties -- the investigator, the surgeon and the patient -- agree to have either one of the pair operated. The side to be done is selected by lot.

The patients are then seen at intervals of six months, one year, and two years. On these visits they undergo standard evaluation for function and radiological examination. Extended follow-up for as long as five years has been completed.

Major Findings:

Thus far it is possible to say that, at 18 months followup, the operated knee was still preferred clinically. The radiological changes have not been impressive in either knee. Twelve patients have been studied to date. It appears that the most important determinant of successful surgery has been the general state of the arthritis.

Significance to Bio-Medical Research and the Program of the Institute:

There has been essentially no controlled work on this important therapeutic modaility which is being widely urged on the basis of inadequate data. There is reason to believe that, almost alone among treatment methods, it will prevent joint damage. This needs to be established.

Proposed Course:

Preliminary analysis of the initial group is underway.

It is proposed to continue to admit suitably selected patients to the study.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-3c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Role of Infection in Rheumatoid Disease

Previous Serial Number: NIAMDD-ARB-1c

Principal Investigator: Dr. John L. Decker

Other Investigators: Robert G. Aptekar, M.D.

Cooperating Units: None

Man Years:

Total:	3/4
Professional:	1/4
Other:	1/2

Project Description:

Objectives:

The overwhelming likelihood is that rheumatoid arthritis is due to an infection. There is a long history of failure in trying to isolate the responsible microbial agent. We therefore believe it important to study the biology of the host-parasite relationship using a known microbial agent to produce an animal disease similar to rheumatoid arthritis. To this purpose we have been assessing the events in the evolution of chronic Mycoplasma hyorhinis arthritis of swine.

Methods Employed:

The polyarthritis which develops following intraperitoneal injection of Mycoplasma hyorhinis has been studied over an 18 month period by: repeated clinical evaluation; serial determination of serum proteins and of serum antibody activity to M. hyorhinis; serial blood cultures; sequential autopsies with material for culture, tissue culture, immunofluorescent studies, and histopathology; sequential synovial fluids for antibody activity.

Major Findings:

In contrast to Yorkshire swine, it has been found that miniature swine (of the Piney Woods strain) develop no chronic arthritis. Histological specimens from control and infected animals extending to 340 days post-inoculation reveal

a transient acute arthritis but no permanent damage and no chronic inflammation. Organisms were not recovered after the 101st day and appear to have been eradicated by the host.

The marked contrast between the two breeds, Piney Woods miniatures as compared to Yorkshire, is reminiscent of discussions of genetic susceptibility of humans to rheumatoid arthritis. The divergent mechanisms by which the animals react to produce these results are of great importance and continue under study.

Proposed Course:

Two manuscripts are now in preparation. No more field work is now planned.

Significance to Bio-Medical Research and the Program of the Institute:

The two strains, one with and the other without chronic arthritis, tend to reinforce the potential relationship to rheumatoid arthritis.

Honors and Awards: None

Publications:

Barden, J. A., Decker, J. L., Dalgard, D. W., Aptekar, R. G.: *Mycoplasma hyorhinis* swine arthritis. III. Modified disease in Piney Woods swine. Infect. Immun. 8:887-890, 1973.

Serial No. NIAMDD-ARB-4c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Tubuloreticular Structures of Circulating Lymphocytes in Systemic Lupus Erythematosus

Previous Serial Number: ARB-4c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Philip M. Grimley (NCI), John H. Klippel, M.D., Dr. Alfred S. Evans (Yale Univ. School of Medicine).

Cooperating Units: National Cancer Institute, Laboratory of Pathology and Department of Epidemiology and Public Health, Yale University School of Medicine.

Man Years:

Total:	1-1/2
Professional:	1
Other:	1/2

Project Description:

Objectives:

Nests of interwoven tubulorecticular structures (TRS) have been recognized by electron microscopy in the cytoplasm of the endothelial cells of kidney and skin from patients with systemic lupus erythematosus for several years now. TRS, also identified in endothelial cells from cases with Sjogren's syndrome, polymyositis and scleroderma, are considered by some to be paramyxovirus particles but others, finding them associated with a number of experimental viral infections, interpret them as nonspecific cellular response to viral infection.

Upon examining a skin biopsy specimen from one of our patients with SLE, TRS were seen not only in the endothelim as expected but also within the cytoplasm of a circulating mononuclear cell, probably a lymphocyte.

The objective of study was to assess the prevalence of TRS in buffy coat lymphocytes from patients with connective tissue disease. This objective is consonant with the larger objective of understanding the pathogenesis of SLE and related diseases.

Methods Employed:

We have continued to prepare and examine lymphocyte specimens from our patients with SLE approximately every 6 months and the results are being compared to measures of disease activity.

Dr. Evans has studied sera of 75 of our subjects measuring the EBV antibody titers by an indirect immunofluorescent technique using the EB3 cell line.

Major Findings:

1. TRS containing lymphocytes have been identified in the cord blood of two normal infants born to mothers with SLE under our care. The number of TRS ⊖ lymphocytes in the cord blood was such that they could not have been maternal lymphocytes unless a totally unknown form of placental concentration is occurring. We believe the finding to be evidence of an effect of maternal antibodies on infant cells; maternal antibodies have frequently been found in cord blood.

2. There was no correlation between the presence of lymphocyte TRS and antibody titers to EBV in the same patient's sera. In our patients the EBV antibody appeared to correlate with immunoglobulin levels of both G and M classes.

3. In our sequential studies, 18 patients with SLE have been studied on more than one occasion over an average span of 8 months. In 14 all studies have been concordant; 9 were always positive and 5 were always negative. In four patients there has been a change; all four were initially positive and became negative. In all four the change was associated with substantial improvement in the patient and a notable decrease in disease activity.

Significance to Bio-Medical Research and the Program of the Institute:

While others have suggested that these structures represent some form of myxovirus nucleocapsid, our evidence suggests that they are secondary to circulating antibody.

Proposed Course:

This work is now being stopped.

Honors and Awards: None

Publications:

Grimley PM, Decker JL, Michelitch HM, Frantz MM: Abnormal structures in circulating lymphocytes from patients with systemic lupus erythematosus and related diseases. Arthritis Rheum 16:313-323 (May-June) 1973.

Klippel JH, Grimley PM, Evans AS, Rothfield NF, Decker JL: Epstein-Barr virus antibody and lymphocyte tubulorecticular structures in systemic lupus erythematosus. Lancet 2:1057-1058 (Nov 10) 1973.

Klippel JH, Grimley PM, Decker JL: Lymphocyte inclusions in newborns of mothers with systemic lupus erythematosus. New Eng J Med 290:96-97 (Jan 10) 1974.

Klippel JH, Grimley PM, Decker JL, Mitchelitch HJ: Lymphocyte tubuloreticular structures - correlation with disease activity in systemic lupus erythematosus. Ann Int Med (In press).

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cellular and humoral immunity in systemic lupus erythematosus (SLE) undergoing randomized cytotoxic therapy.

Previous Serial Number: 6c

Principal Investigator: John L. Decker, M.D.

Other Investigators: Alfred D. Steinberg, M.D., Robert G. Aptekar, M.D., and Thomas A. Waldmann, M.D., (C:MET)

Cooperating Units: National Cancer Institute, Metabolism Branch.

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

The literature provides a variety of statements as to the capacity of patients with SLE to respond to antigens which stimulate humoral and delayed hypersensitivity responses. There is evidence that these are altered by the disease itself (especially when active) but such studies are always perturbed by the use or the non-use of adrenal corticosteroid therapy which is essential to the patients' well-being. Virtually nothing is known of the effect, on this background, of the cytotoxic drugs azathioprine and cyclophosphamide. We designed a study to answer the question do azathioprine or cyclophosphamide, given in clinically useful doses, actually produce immunosuppression in SLE as contrasted to the effects of placebo?

Methods Employed:

Twenty-five patients were evaluated by skin tests to assess delayed hypersensitivity and by circulating antibody titers to assess humoral immunity before and during a controlled trial of the use of prednisone alone or in combination with cyclophosphamide or azathioprine. Standard skin test preparations of PPD, mumps, SK/SD, candida, trochophyton and mixed respiratory vaccine were used. Purified polysaccharide antigens of pneumococcus Type I (Pn I) (given before study), Type II (Pn II), the Vi antigen of E.coli, KLH and BRU (all given during study) were used to stimulate circulating immunity.

Major Findings:

Skin test results were not appreciably different in the 3 treatment groups. None of 7 patients on prednisone could be sensitized to DNCB but 3 of 8 were positive on azathioprine and 1 of 3 on cyclophosphamide.

Mean antibody levels to all antigens except Vi were the same in all 3 groups and were lower than non-SLE controls. The response to Vi was significantly depressed only in patients on azathioprine.

Significance to Bio-Medical Research and the Program of the Institute:

Drug therapy apparently facilitates the cellular reaction to DNCB. This may be important in improving the prognosis of patients with SLE in terms of decreasing the likelihood of infection. Impairment of cellular immunity appeared to be greater than that of humoral immunity.

Proposed Course:

This study has been completed and a manuscript is in preparation.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-6c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mitral valve disease of systemic lupus erythematosus: A cause of severe congestive heart failure reversed by valve replacement

Previous Serial Number: None

Principal Investigator: Dr. Stephen Paget

Other Investigators: Dr. Bernadine Bulkley (NHLI-P), Dr. Leonard Grauer (NHLI-C), and Dr. R. Seningen (CC-R)

Cooperating Units: National Heart and Lung Institute: Section of Pathology, Division of Cardiology and Clinical Center - Dept. of Diagnostic Radiology

Man Years:

Total:	3/4
Professional:	1/2
Other:	1/4

Project Description:

Objectives:

Case report described a patient with severe mitral valve disease due to Libman-Sacks endocarditis; that lesion has previously been known to only rarely lead to significant valvular dysfunction. A review of the literature on this topic was also made and reported. Further review of the literature and echocardiographic studies were undertaken to further evaluate the incidence of valvular dysfunction in patients with SLE with early detection of potentially significant abnormalities.

Methods Employed:

The case report included the following diagnostic tests in evaluating the etiology and extent of the valvular lesion which necessitated surgery: Echocardiography, angiocardiography and catheterization studies, pathologic evaluation, plain X-ray and surgical evaluation of the lesion. In the follow-up echocardiography study, patients with any type of murmur are evaluated with CXR, phono (if the murmur is difficult to characterize) and echocardiogram.

Major Findings:

The case report showed that Libman-Sacks endocarditis, a lesion which has previously only been diagnosed at autopsy, was the cause of significant valvular dysfunction and led to severe congestive heart failure; the valvular disorder was corrected surgically - this was the first such report in the literature which described such a lesion and surgical correction. With prolonged life-span in patients with SLE, this lesion might become a more common complication which can lead to severe valvular dysfunction. In our follow-up study, no significant valvular abnormalities have been noted in the five patients evaluated.

Significance to Bio-Medical Research and the Program of the Institute:

The case report alerts physicians caring for patients with SLE that the Libman-Sacks endocardial lesion can lead to severe valvular dysfunction and congestive heart failure; this is specifically important because most physicians feel that the incidence of the lesion in decreasing in this age of steroids and that they are only of autopsy interest. It also stresses the fact that a pre-operative diagnosis can be made with the use of echocardiography (a non-invasive test) and angiocardiography. This is the first case report describing a patient in whom the diagnosis of Libman-Sacks endocarditis was made prior to autopsy; we also described the first report of a surgically corrected lesion and also the largest such lesion ever found pre or post mortem.

Proposed Course:

Case report has been completed and led to a publication. Echocardiographic studies are ongoing.

Honors and Awards: None

Publications:

Paget SA, Bulkley, BH, Grauer, LE and Seningen, R: Mitral valve disease of systemic lupus erythematosus: A cause of severe congestive heart failure reversed by valve replacement Am. J Med (In Press).

Serial No. NIAMDD-ARB- 7c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Leukopenia in Connective Tissue Disease

Previous Serial Number: ARB-19c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Sheldon Wolff (I-LCI) and Dr. Harry Kimball (I-LCI)

Cooperating Units: National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation

Proposed Course:

This project has been completed and led to a publication.

Publication:

Kimball HR, Wolff SM, Talal N, Plotz PH, Decker JL: Marrow granulocyte reserves in the rheumatic diseases. Arthritis Rheum 16:345-352, 1973

Serial No. NIAMDD-ARB-8c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Infection in Systemic Lupus Erythematosus

Previous Serial Number: ARB-18c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Parker J. Staples (Strong Memorial Hospital), Dr. Robert S. Gordon (A:CI), and Dr. Dale Gerding (CR-OD)

Cooperating Units: Strong Memorial Hospital, Rochester, New York; NIAMDD, Clinical Investigations; Division of Computer Research and Technology, Office of the Director

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This project has been completed and led to a publication.

Publication:

Staples PJ, Gerding DN, Decker JL, Gordon RS: Incidence of infection in systemic lupus erythematosus. Arthritis Rheum 17:1-10, 1974

Serial No. NIAMDD-ARB-9c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the pathophysiology of nervous system involvement in systemic lupus erythematosus (SLE)

Previous Serial Number: ARB-5c

Principal Investigator: Dr. Nortin M. Hadler

Other Investigators: Dr. Michael Frank (I:LCI), Dr. Robert Gerwin (I:LCI), Dr. John Whitaker (N:IR), Dr. Max Baker (M:DCBR), Dr. Edward Donnelly (M:AP) and Dr. John L. Decker

Cooperating Units: National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigations; National Institute of Neurological Diseases and Stroke, Intramural Research; National Institute of Mental Health, Division of Clinical and Behavioral Research

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

This project has been completed and led to three publications.

Publications:

1. Hadler NM, Gerwin RD, Whitaker JN, Baker M, Decker JL: The fourth component of complement in the cerebrospinal fluid in systemic lupus erythematosus. Arthritis Rheum 16:507-521, 1973

2. Baker M, Hadler NM, Whitaker JN, Dunner DL, Gerwin RD, Decker JL: Psychopahtology in systemic lupus erythematosus. II. Relation to clinical observations, corticosteroid administration and cerebrospinal fluid C4. Seminars in Arthritis and Rheumatism 3:111-126, 1973

3. Baker M: Psychopathology in systemic lupus erythematosus. I. Psychiatric observations. Seminars in Arthritis and Rheumatism 3:95-110, 1973

Serial No. NIAMDD-ARB- 10c
1. Arthritis and Rheumatsim
2. Connective Tissue Disease
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Systemic Lupus Erythematosus Coagulation Study

Previous Serial Number: NIAMDD-ARB-7c

Principal Investigator: Dr. John L. Decker

Other Investigators: Dr. Alfred D. Steinberg, Dr. Harvey Gralnick (CC-CP), Dr. Sally Marchesi (CC-CH), and Dr. Robert Aptekar

Cooperating Units: Clinical Center, Clinical Pathology Department

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives:

To study abnormalities of coagulation in patients with systemic lupus erythematosus (SLE), and to correlate these abnormalities with disease activity.

Methods Employed:

Patients with SLE who are admitted to the Clinical Center and selected patients with SLE seen in the outpatient department are studied.

Evaluation of Clinical status is done independently by Drs. Decker and Steinberg and the results recorded on separate evaluation sheets. On the same day, the patient's blood is studied for coagulation factors, fibrin split products, and other hematological parameters. A 24-hour urine is also studied for fibrin split products. The coagulation study by clinical hematology is performed without knowledge of the clinical evaluation.

Major Findings:

Patients with active nephritis had elevated fibrin degradation products (FDP) excretions ranging from 1 to 50 mg. per 24 hours and the highest excretions were generally found in those with most active disease.

However, sequential assessment of 19 patients showed that FDP excretion fluctuated widely and without apparent relationship to disease. The measure was not correlated with creatinine clearance, urinary protein, serum anti-DNA antibodies, nor serum complement values.

Significance to Bio-Medical Research and the Program of the Institute:

We concluded that the measure was not of clinical merit at our present level of comprehension.

Proposed Course:

This project has been completed and led to a publication.

Honors and Awards: None

Publications:

Marchesi SL, Aptekar RG, Steinberg AD, Gralnick HR, Decker JL: Urinary fibrin split products in lupus nephritis; correlation with other parameters of renal disease. Arthritis Rheum 17:158-164, 1974.

Serial No. NIAMDD-ARB-11c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Characterization of infiltrating lymphocytes in rheumatoid synovial membranes.

Previous Serial Number: None

Principal Investigator: Dr. John A. van Boxel

Other Investigators: Dr. Stephen Paget
Dr. John L. Decker

Cooperating Units: None

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

Immunological mechanisms in the pathogenesis of rheumatoid arthritis may be revealed by study of the remarkably dense lymphocytic infiltrations in rheumatoid synovia. Lymphocytes have the capacity to damage tissue (cytotoxicity) and secrete lymphokines and this can be assessed *in vitro*. They can be classified in B and T subclasses on the basis of surface membrane markers.

Methods Employed:

A method was developed for the extraction and purification of the infiltrating lymphocytes. Synovial tissue is dissociated with collagenase and the single cell suspension fractionated by velocity sedimentation. This technique exploits the marked difference in size between lymphocytes and fibroblasts, the rate of sedimentation being directly proportional to the square of the radius of the cells. A lymphocyte yield of approximately 70% was obtained with 95% viability. Lymphocyte subclasses were identified using antisera to the immunoglobulin subclasses (B cells) and rosette formation using neuraminidase treated sheep erythrocytes (T cell marker).

Major Findings:

Preliminary data on three synovia examined indicates that the majority (60-70%) of the lymphocytes are T cells.

Significance to Bio-Medical Research and the Program of the Institute:

There is much evidence for immunological mechanisms having a role in the pathogenesis of rheumatoid arthritis. A striking feature of rheumatoid synovial membranes is the extremely dense accumulation of lymphocytes. Recent in vitro studies reveal that lymphocytes have the capacity to damage tissue. Study of these intralesional lymphocytes may help to determine whether an autoimmune reaction is taking place.

Proposed Course:

The method described can be performed under sterile conditions. These lymphocytes will therefore be tested for their cytotoxic capacity. Attempts will be made to assess whether they can destroy the fractionated synovial cells in vitro, a direct demonstration of their pathogenetic role.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB- 12c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Binding of Aggregated γ-globulin to T Lymphocytes

Previous Serial Number: None

Principal Investigator: John A. van Boxel, M.D.

Other Investigators: David L. Rosenstreich, M.D.

Cooperating Units: Laboratory of Microbiology and Immunology, National Institute of Dental Research, NIH

Man Years:

Total:	1/2
Professional:	1/4
Othe;r	1/4

Project Description:

Objectives:

Aggregated γ-globulin and antigen-antibody complexes have been shown to bind to the surface membrane of bone marrow derived or B lymphocytes and this reaction is used as a marker for this class of cell. A membrane receptor for the Fc portion of the γ-globulin molecule is believed to be responsible for this binding. Our studies have been to further characterize this receptor in the guinea pig.

Methods Employed:

Binding of heat aggregated γ-globulin was demonstrated with fluorescence microscopy of living cells using fluorochrome tagged antisera. The indirect method that was developed afforded a technique of sufficient sensitivity to detect aggregates bound to activated T cells, these being finer and less densely distributed than on B cells. These cells were further characterized by double and triple labelling with a B cell marker (surface immunoglobulin) and a T cell marker (modification of the procedure using spontaneous E-rosette formation with rabbit erythrocytes resulted in this marker being specific for T cells. These methods were applied to the examination of different lymphoid populations and to sensitized cells after specific antigen activation in vitro.

Major Findings:

We have found that a subpopulation of thymus-derived or T lymphocytes, the other major lymphocyte class, have the capacity to bind aggregated γ-globulin, a property hitherto believed to be exclusive to the B class of lymphocyte. Such cells could be identified in substantial number in guinea pig draining lymph nodes and in high frequency (>50%) in an inflammatory (peritoneal) exudate. We have demonstrated that cells sensitized to DNP-Ovalbumin develop the capacity to bind aggregated γ-globulin upon activation by antigen stimulation. This finding strongly suggests that the subpopulation of T lymphocytes binding aggregates identifies those T cells that have undergone activation.

Significance to Bio-Medical Research and the Program of the Institute:

These findings suggest that T cells acquire a receptor for the Fc portion of the γ-globulin molecule upon antigen activation with the capacity to bind antigen-antibody complexes and aggregated γ-globulin. (1) This receptor may have a key role in the antigen T cell-B cell-macrophage interaction involved in the antigen processing stage of the immune response. (2) This finding has direct bearing on the current contentious question of the existence or non-existence of T cell immunoglobulins; such immunoglobulins may well be present due to the passive absorption of aggregated protein via this Fc receptor. (3) Our demonstration that activated T cells can be identified by double labelling with aggregated γ-globulin and spontaneous E-rosette formation using rabbit erythrocytes provides a potentially useful marker for the identification of activated T cells in mixed lymphoid populations. This is of special significance on account of the probable major biological roles of the activated T lymphocyte; activated T lymphocytes have greatly enhanced effector (killer) functions and have an enhanced secretion of lymphokines.

Proposed Course:

1. We will attempt to verify these findings in man; preliminary results indicate that this is possible. Our approach is to activate cells in vitro using the mixed lymphocyte reaction (MLR) and then to test these cells for their capacity to bind aggregates as well as the degree of activation by measuring [H^3] thymidine incorporation. 2. Using the marker described above we will examine circulating as well as intralesional lymphoid populations in various autoimmune diseases for the presence of activated T cells eg peripheral blood in SLE, synovial fluid and synovium in R.A. 3. We will assess whether activated T cells have the capacity to mediate antibody-dependent cytotoxicity on account of the known role of this receptor in this reaction.

Honors and Awards: None

Publications:

van Boxel JA, Rosenstreich DL: Binding of aggregated γ-globulin to activated T lymphocytes in the guinea pig. J Exp Med 139-1002-1014, 1974

Serial No. NIAMDD-ARB-13c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cell Surface Immunoglobulins

Previous Serial Number: None

Principal Investigator: John A. van Boxel, M.D.

Other Investigators: Donald Buell, M.D.

Cooperating Units: National Cancer Institute, Immunology Branch

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

We have previously demonstrated that IgD, an immunoglobulin class of as yet unknown function, is bound to the membrane of an unexpectedly high frequency of human peripheral blood lymphocytes. We have since focussed on the question of whether IgD is produced by the cell that bears it or is adherent because of cytophilic properties. We have also previously shown that individual peripheral blood lymphocytes of some patients with Sjogren's syndrome bear multiple immunoglobulin classes in contrast to the single immunoglobulin class usually found in normals. Several mechanisms could account for this phenomenon. It is of interest that multiple immunoglobulin classes have also been found in patients with leukemia and lymphoma because of the spectrum of benign to malignant lymphoproliferation in Sjogren's syndrome.

Methods Employed:

We have examined a number of long-term human lymphoid cell lines for the presence of surface immunoglobulin using fluorochrome-tagged antisera and purified antibodies. Secreted immunoglobulin was measured by radioimmunoelectrophoresis. Studies on the redistribution of cell surface immunoglobulin (capping) were performed using anti-immunoglobulin antisera conjugated with different fluorochromes.

Major Findings:

1. We have found that IgD is bound to the membrane of a relatively large proportion of human lymphoid cell lines. This finding alone provides almost conclusive evidence that IgD is produced by the cell that bears it, since these cell lines have no contact with exogenous human immunoglobulin and examination of the supernates revealed no secreted IgD. These cell lines also provide a tool for the in vitro investigation of cell associated IgD.

2. The cell lines found to bear IgD also had surface IgG, IgM and IgA but not IgE and only a single light chain, kappa or lambda. Several of the non-IgD bearing cell lines also bore multiple classes, and two cell lines bore both kappa and lambda light chains. Antibody-induced aggregation (capping) studies revealed independent migration of the heavy chains. By contrast caps induced by antibodies to light chains included all surface heavy chains, suggesting that these heavy chains occur in the form of discrete immunoglobulin molecules. Despite multiple surface immunoglobulin classes, the cells secreted only a single class, a finding consistent with the concept that cell surface and secreted immunoglobulin are regulated by separate mechanisms.

Significance to Bio-Medical Research and the Program of the Institute:

Since our finding of IgD on a large proportion of adult lymphocytes, IgD has been shown to be the predominant surface immunoglobulin in the neonate and in addition those cells bearing IgD also bear IgM. This suggests a key biological role for cell-associated IgD. Our evidence that IgD is produced by the cell that bears it suggests that it functions as a receptor. The finding of multiple immunoglobulin classes indicates that autoantibodies to B cells or cytophilic antibodies need not be evoked to explain the finding of multiple immunoglobulin classes in clinical states, in which this abnormality may represent dedifferentiation of the malignant or abnormal cells which permits the expression of multiple Ig classes similar to that seen in these human cell lines.

Proposed Course:

1. We have been able (with Dr. Henry Metzger) to fractionate purified antibody to an IgD myeloma of a local patient. Studies with this reagent will include investigation to determine whether cells bearing both surface IgD and IgM can be stimulated to undergo transformation using antibodies to IgD and IgM.

2. Studies are in progress to determine whether or not the multiple immunoglobulin classes on these cells share idiotypic specificities.

Honors and Awards: None

Publications:

1. van Boxel JA, Paul WE, Terry W., Green I: IgD bearing human lymphocytes. J Immunol 109:648-651, 1972

2. van Boxel JA, Hardin JA, Green I, Paul WE: Multiple heavy chain determinants on individual B lymphocytes in the peripheral blood of patients with Sjögren's syndrome. New Eng J Med 289:823-827, 1973

Serial No. NIAMDD-ARB-14c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Antibody-dependent lymphoid cell mediated cytotoxicity

Previous Serial Number: None

Principal Investigator: John A. van Boxel, M.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

Tissue injury mediated by lymphocytes (cytotoxicity) is currently believed to be of prime importance in autoimmune diseases, and tumor and graft rejection. Two in vitro models of this reaction are recognized (1) mediated by sensitized T lymphocytes, (2) mediated by non-sensitized lymphocytes when target cells are coated by antibody (antibody-dependent lymphoid cell mediated cytotoxicity). We have previously shown that the latter model (ADLC) does not require the presence of T lymphocytes (Science 175:194, 1972). We have further provided evidence that the effector cells in ADLC mediated by mouse spleen cells form a subclass of B lymphocytes that bear a Mg^{++} independent complement receptor. We are continuing with studies to more precisely identify the effector cell. Regarding the mechanism of this reaction, we have found that neither the classical nor the alternate complement pathways have a role in cytotoxicity and that although the complement receptor is not involved, the Fc or aggregate receptor on the cell surface has a critical role in effector-target interaction. We have since shown (see aggregate binding to T cells) that T lymphocytes, when activated, appear to develop an Fc receptor. This raises the question as to whether activated T cells may also kill target cells in this system.

Methods Employed:

Our assay involves radiolabelled (Cr^{51}) target red cells and mouse spleen effector cells, incubated together for 24 hours. Both gradient density and velocity sedimentation are used to fractionate spleen cells.

Major Findings:

Preliminary data show that activated T cells, despite the development of an Fc receptor, have no role in this reaction. Thymus cells, which do not have killer function, were activated in the mixed lymphocyte reaction (i.e., Balb/C and C57/B16 in mixed culture for 3 days) using the recent modified culture system of Peck and Bach, and were tested for cytotoxic activity, capacity to bind aggregate and [H^3] incorporation.

Significance to Bio-Medical Research and the Program of the Institute:

Because lymphocytes appear to have an important role in many pathological processes, knowledge of the identity and mechanism of action of the effector cell(s) may lead to an understanding of their role in vivo and to the eventual manipulation of these reactions.

Proposed Course:

Studies will be continued to identify the killer cell in our system and to clarify the mechanism of this reaction. We will also attempt to assess the role of this in vitro phenomenon in vivo. One approach will be to test lymphocytes infiltrating lesions in autoimmune diseases (viz synovium, extracted as described above), for their capacity to mediate antibody dependent cytotoxicity.

Honors and Awards: None

Publications:

1. van Boxel JA,Stobo J, Paul WE, Green I: Antibody-dependent lymphoid cell-mediated cytotoxicity: No requirement for thymus derived lymphocytes. Science 175:194-196, 1972

2. van Boxel JA, Paul WE, Frank MM, Green I: Antibody-dependent lymphoid cell mediated cytotoxicity: role of lymphocytes bearing a receptor for complement. J Immunol 110:1028-1036, 1973

3. van Boxel JA, Paul WE, Green I, Frank MM: Antibody dependent lymphoid cell mediated cytotoxicity: role of complement. J Immunol 112:398, 1974

Serial No. NIAMDD-ARB-15c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Aspirin Hepatotoxicity in Patients with Collagen Vascular Disease

Previous Serial Number: None

Principal Investigator: William E. Seaman, M.D.

Other Investigators: Paul H. Plotz, M.D.

Cooperating Units: None

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

Aspirin hepatotoxicity has been noted in patients with acute rheumatic fever, juvenile rheumatoid arthritis, and rheumatoid arthritis. We determined to study this phenomenon in patients with lupus erythematosus and subsequently in patients with rheumatoid arthritis. The goals were to gain an understanding of the frequency of this phenomenon, to determine if hepatotoxicity occurred independently of disease activity, and to gain some understanding of the doses of aspirin on which hepatotoxicity occurred.

Methods Employed:

Our studies have been divided into three areas. The first is observational: Three patients with lupus erythematosus were observed to have abnormal liver tests while taking aspirin. The tests returned to normal when aspirin was stopped and recurred when the patients were rechallenged with aspirin. A liver biopsy in one patient was consistent with chronic active hepatitis and a biopsy from a second patient showed acute hepatocellular injury. In neither case have abnormal liver tests recurred off aspirin. Following these 3 patients, we have observed 8 more patients who developed abnormal liver tests while taking aspirin, and whose tests returned to normal when aspirin was stopped. Because of our observations on the first 3 patients, the second

phase of study was initiated which consisted of a prospective in-hospital study in which patients with lupus erythematosus or rheumatoid arthritis were given aspirin (starting with 50 mg/kg per day and increased by 600 mg every other day) until the morning salicylate level was 25-30 mg%, which was then maintained for two weeks. If abnormal liver tests developed, the aspirin was stopped. The third aspect of our work consisted of a retrospective study of all in-patients with lupus erythematosus over a two and one-half year period. Eighteen additional patients with abnormal transaminase levels were identified. A chart review was conducted to determine if these were related to aspirin. A fourth phase of the study, an in-hospital study of the effects of aspirin in normal volunteers was initiated, but was discontinued at the advice of the Medical Board when the third volunteer developed abnormal transaminase levels on aspirin.

Major Findings:

In the in-hospital study, 3 of 18 patients with rheumatoid arthritis and 5 of 14 patients with lupus erythematosus developed hepatotoxicity while taking aspirin. Hepatotoxicity was manifested by an elevation in SGOT and/or SGPT and in 2 cases by clinical evidence of hepatitis. In all cases abnormal tests rapidly returned to normal as soon as aspirin was stopped. The 3 patients with rheumatoid arthritis who developed hetatotoxicity, did so only on high doses and at the end of the study. In contrast, 3 of the 5 patients with lupus erythematosus who developed abnormal tests did so with low dise (salicylate levels 12-30mg%). Among patients with lupus erythematosus, hepatotoxicity appeared to develop in those patients who had active diseases; 4 of the 5 patients with active disease developed hepatotoxicity, while only 1 of the 9 patients with inactive disease did. During the course of this study, 8 patients with lupus erythematosus and aspirin hepatotoxicity (who were not involved in this study) were identified. All of these patients also had active disease, though in this uncontrolled situation, only patients with active disease would be likely to be taking much aspirin.

In the retrospective study of all in-patients with lupus over a 2-1/2 year period 18 additional patients with abnormal transaminase levels were identified. The etiology in 5 of these patients were: alcohol, isoniazid, serum hepatitis (HB Ag positive)troleandomycin, azathioprine. All of the remaining 13 patients developed abnormal test within 3 weeks of starting aspirin or having the dose increased. All 13 of these patients had active disease.

In a study involving normal volunteers, one normal volunteer developed mildly abnormal SGPT levels while taking high-dose aspirin (serum salicylate 25 mg%). As with almost all patients studied by ourselves or others, the abnormal tests rapidly returned to normal on cessation of aspirin.

Significance to Bio-Medical Research and the Program of the Institute:

Following the recognition of aspirin hepatotoxicity in patients with lupus, it has become apparent that this is startingly common among patients with active disease. The retrospective study indicates, in fact, that abnormal liver tests in lupus are usually due to drugs, and that aspirin is the most common cause. Liver biopsies from two patients with aspirin hepatotoxicity have been obtained, and one showed chronic active hepatitis. This leads us to wonder if chronic aspirin ingestion may be an occult cause of chronic liver disease in the population. Although in patients with lupus, the development of hepatotoxicity appears to depend on disease activity, the observation that aspirin hepatotoxicity could occur in a normal volunteer further supports this possibility. The resolution of this question will require large epidemiologic studies, which we hope our studies will instigate.

Proposed Course:

The formal investigation of aspirin hepatotoxicity at the National Institutes of Health will probably end with the departure of the Principal Investigator on July 1, 1974, though some studies may be carried on.

Honors and Awards: None

Publications:

Seaman WE, Ishak KG, Plotz PH: Aspirin-induced hepatotoxicity in patients with systemic lupus erythematosus. Ann Int Med 80:1-8, 1974

Serial No. NIAMDD-ARB- 16c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: **The removal of bilirubin and other albumin bound substances from blood**

Previous Serial Number: **ARB-14c**

Principal Investigator: **Dr. Paul H. Plotz**

Other Investigators: **Mrs. Kay Gordon, Dr. Bruce Scharschmidt (A:DDB), Dr. Paul Berk (A:DDB), Mr. John Vergalla (A:DDB), and Ms. Jean Waggoner (A:DDB)**

Cooperating Units: **Digestive Diseases Branch, NIAMDD**

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

Substances such as bilirubin that binding tightly to plasma protein cannot be removed by dialysis. We have attempted to remove such substances by employing affinity chromatography on protein - agarose beads.

Methods Employed:

Human albumin is conjugated to agarose: the resulting beads are placed in a column and plasma or whole blood, are passed over the column. Bilirubin or other substances are assayed before and after passage. The bound material is eluted and assayed.

Major Findings:

Agarose-albumin columns can bind in excess of 150 µg bilirubin per gram of wet weight of gel. The BR can be completely eluted and the gel reused. The gel material is compatible with blood and can be used in a perfusion system with living rats. Thyroxine and salicylate bind to such beads too.

Significance to Bio-Medical Research and the Program of the Institute:

Such conjugates may be useful for removing bilirubin and other protein-bound toxic substances from human blood.

Proposed Course:

Further studies will include investigation of the binding of other substances to agarose-protein beads and the preparation of materials safe for in vitro use. In vivo studies on rats, monkeys, and sheep have begun.

Honors and Awards: None

Publications:

1. Plotz PH, Berk PD, Scharschmidt BF, Gordon JK, Vergalla J: Removing substances from blood by affinity chromatography. I. Removing bilirubin and other albumin-bound substances from plasma and blood with albumin-conjugated agarose beads. J Clin Invest 53:778-785, 1974

2. Scharschmidt BF, Plotz PH, Berk PD, Waggoner JG, Vergalla J: Removing substances from blood by affinity chromatography. II. Removing bilirubin from the blood of jaundiced rats by hemoperfusion over albumin-conjugated agarose beads. J Clin Invest 53:786-795, 1974

Serial No. NIAMDD-ARB-17c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effects of alloantisera on recognition of antigen by T cells

Previous Serial Number: None

Principal Investigator: Dr. William E. Seaman

Other Investigators: Dr. William Paul

Cooperating Units: NIAID - Laboratory of Immunology

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

In vitro studies in guinea pigs have suggested that the cellular surface markers determining histocompatability specificity are associated with the recognition of antigen by lymphocytes. It would be useful to study this phenomenon in mice, where inbred strains are more plentiful and a wider range of genetically controlled antigens has been identified. However, in vitro tests of the immune response in mice have been erratic. The present study was designed to allow testing in vivo.

Methods Employed:

Mice from two inbred strains, one able to respond to the polymer TGAL and the other not, were bred to provide F_1 offspring (all of whom respond). These offspring were immunized either with TGAL or dinitrophenol-bovine serum albumin (DNP-BSA). Other offspring were irradiated and given cells from animals immunized with TGAL and from animals immunized with DNP-BSA. Stimulation of these animals with DNP-TGAL led to a secondary immune response. The experiment planned was to repeat the above control experiment, but expose the cells from animals immunized with TGAL to highly radioactive, iodinated TGAL ($T^{125}IGAL$). This labeled antigen should stick to those cells bearing receptors for TGAL and radiate them, abnegating the immune response (cell "suicide"). It was then planned to study the effects of antisera to the two parental strains on this cell suicide, to see if recognition of the antigen was blocked by antisera to the responder parent as opposed to antisera to the nonresponder parent.

Major Findings:

Many technical difficulties were encountered, but the major obstacle that would not be overcome was the inability to abnegate the immune response with hot antigen. Other workers attempting the same experiment in guinea pigs met with the same difficulty.

Significance to Bio-Medical Research and the Program of the Institute:

Regulation of the immune response appears to be of major importance in the growth of tumors and probably in most of the "collagen vascular diseases", as well as in resistance to infection. An understanding of the cellular mechanisms underlying control of the immune response is expected to be of practical benefit.

Proposed Course:

This study has been dropped because of the difficulties cited above.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-18c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Therapy of SLE: A controlled trial comparing high dose intravenous cyclophosphamide with placebo

Previous Serial Number: ARB-9c

Principal Investigator: Dr. Paul H. Plotz

Other Investigators: Dr. John L. Decker and Dr. Alfred D. Steinberg

Cooperating Units: None

Man Years:

Total:	1-1/2
Professional:	1
Other:	1/2

Project Description:

Objectives:

At present no satisfactory therapy exists for severe manifestation of SLE except for renal disease. Even renal disease does not respond well to conventional therapy when the creatinine clearance is markedly reduced. In this study we will test the efficacy of high dose intravenous therapy with cyclophosphamide for the control of severe SLE unresponsive to a trial of high dose of prednisone (up to 2 mg per kg for 3 weeks).

Methods Employed:

Patients with 1) severe renal disease or rapid deterioration of renal function, 2) sensitivity to azathioprine precluding the CAP protocol, 3) severe extra-renal lupus (seizures, hemorrhage, psychosis, hemolytic anemia, myocarditis, or pericarditis uncontrolled by high-dose prednisone) will be randomly assigned to receive intravenous cyclophosphamide or placebo for 3 initial injections over 6 weeks and then at 3 month intervals. Renal and non-renal patients will be separately randomized. The study will be double-blind: neither physicians nor patients will know which therapy is being administered. The end point of the study will be death or the need for renal dialysis except that patients well for 1 year may have therapy stopped. The potential hazards and benefits will be explained in full to all patients or their families.

Major Findings:

Six patients have been admitted to the study so far - two in the non-renal group and four in the renal group. No findings of significance to date.

Significance to Bio-Medical Research and the Program of the Institute:

The therapy of SLE has been under study here for several years. The present study is an attempt to extend the therapy for patients unresponsive to conventional therapy.

Proposed Course:

We plan to enter 20 to 30 patients into the study over the next 3-5 years.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-19c
1. Arthritis and Rheumatism
2. Connective Tissue Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Controlled study of cyclophosphamide and azathioprine in systemic lupus erythematosus with nephritis

Previous Serial Number: 8C

Principal Investigators: Dr. John L. Decker and Dr. Alfred D. Steinberg

Other Investigators: Dr. Paul H. Plotz, Dr. Jack Klippel, Dr. Robert Aptekar

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

Two previous ten week studies by our branch suggested that cyclophosphamide was effective in the treatment of lupus nephritis and that azathioprine was less effective. A long-term study of these patients is being carried out to determine whether or not one therapeutic program is superior over the course of several years. In addition, newer drug programs have been suggested by study in New Zealand mice.

Methods Employed:

Patients meeting predefined criteria for diagnosis of SLE and activity of nephritis will be admitted to the trial. In a baseline control period, the patients will be kept on the anti-inflammatory regimen (usually prednisone and aspirin) needed to control extra-renal manifestations. These will be continued into the study period with as little change as possible and with the addition of randomly assigned 1) cyclophosphamide, 2) azathioprine, 3) placebo, 4) cyclophosphamide plus azathioprine, 5) intravenous cyclophosphamide.

A large number of serological, chemical and urine values will be serially obtained and the patients will be followed for life. Immunoglobulin levels, anti-DNA antibodies, and serum complement are among those

measures. We will determine time to death or renal dialysis in each treatment program and assess the toxicity as well as efficacy of each program.

Major Findings:

Patients receiving no immunosuppressive drug have progressed to death or dialysis at a rate faster than those receiving drug. Significant differences have not yet been reached between any drug programs. Toxicity from cyclophosphamide appears to be similar in frequency and severity to that from azathioprine.

Significance to Bio-Medical Research and the Program of the Institute:

If differences in the response of the disease and some of the immunological processes can be detected between different drug programs, it will be possible to draw conclusions with reference to the cell populations involved in systemic lupus erythematosus and thus increase our understanding of the disease process itself. It is expected that the results will permit us to select among treatment programs in clinical management.

Proposed Course:

A long-term followup of these patients will be continued, primarily by one-week admissions every 6 months.

Honors and Awards: None

Publications:

1. Steinberg AD: An approach to the use of immunosuppressive drugs in non-malignant diseases. J. Allergy & Clin. Immunol. 52:242-250, 1973.

2. Aptekar RG, Atkinson JP, Decker JL, Wolff SM, Chu EW: Bladder toxicity with chronic oral cyclophosphamide therapy in non-malignant disease. Arthritis Rheum. 16:461-467, 1973.

3. Gershwin ME, Goetzl E, Steinberg AD: Cyclophosphamide. Use in clinical practice. Annals Intern. Med. 80:531-540, 1974.

4. Steinberg AD, Decker JL: A double blind controlled trial comparing cyclophosphamide, azathioprine and placebo in the treatment of lupus glomerulonephritis. Arthritis Rheum., in press.

5. Aptekar RG, Atkinson JP, Decker JL, Wolff SM, Chu EW: Bladder toxicity with chronic oral cyclophosphamide therapy in non-malignant disease. Arthritis Rheum. 16:461-467, 1973.

6. Aptekar RG, Lawless OJ, Decker JL: Deforming non-erosive hand arthritis in systemic lupus erythematosus. Clin. Orthop, in press.

7. Clark RA, Kimball HR, Decker JL: Neutrophil chemotaxis in systemic lupus erythematosus. Ann. Rheu. Dis., in press.

8. Klippel JH, Decker JL: Relative macrocytosis in cyclophosphamide and azathioprine therpay. JAMA, in press.

9. Aptekar RG, Klippel JH, Becker KE, Carson DA, Seaman SW, Decker JL: Avascular necrosis of the talus, scaphoid, and metatarsal head in systemic lupus erythematosus. Clin. Orthop, in press.

Serial No. NIAMDD-ARB-20c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Antibodies to Double Stranded DNA and RNA in Spontaneous and Drug-Induced Systemic Lupus Erythematosus and other Diseases

Previous Serial Number: 10C

Principal Investigator: Dr. Alfred Steinberg

Other Investigators: Dr. Merrill Gershwin, Dr. Naomi Gerber, Dr. Irwin Scher (NNMC)

Cooperating Units: National Naval Medical Center

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

Study the characteristics of anti-DNA and anti-RNA antibodies in human and murine lupus. Determine genetic contributions to the immune response to nucleic acid antigens.

Methods Employed:

Farr ammonium sulfate precipitation assay, using single or double stranded C^{14}-KB cell DNA, or C^{14} polyinosinic·polycytidylic acid as antigen. Standard precipitin reaction using labeled and unlabeled nucleic acids. Standard immunization procedures.

Major Findings.

1. Humans with SLE and renal disease have antibodies to DNA with greater avidity than do patients with SLE without renal disease. Precipitating antibodies to DNA occur only in patients with renal disease.

2. An X-linked immune response gene controls the antibody response to double stranded RNA. Preliminary study reveals high (NZB and ALN) and low (DBA/2 and BALB/c) responding strains to immunization with single stranded DNA.

Significance to Bio-Medical Research and the Program of the Institute:

Genetic studies may reveal one variable in the pathogenesis of antinuclear antibodies as well as explain their occurrence primarily in females.

Qualitative aspects of antinuclear antibodies may explain why some patients develop renal disease and others do not. This enables us to better define patients at risk earlier in the course of their illness which will lead to more vigorous treatment of patients at greater risk and less vigorous treatment of patients at lesser risk.

Proposed Course:

Define the genetics of the antibody response to DNA. Further study the characteristics of antinuclear antibodies.

Honors and Awards: None

Publications:

1. Gershwin ME, Steinberg AD: Qualitative characteristics of anti-DNA antibodies in lupus nephritis. Arthritis Rheum., in press.

Serial No. NIAMDD-ARB-21c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Pathogenesis of Autoimmunity in New Zealand Mice

Previous Serial Number: 12C

Principal Investigator: Dr. Alfred D. Steinberg

Other Investigators: Dr. Naomi L. Gerber, Dr. Merrill E. Gershwin, Dr. Michael Gelfand (NIAID), Dr. Irwin Scher (NNMC), Dr. Adi Gazdar (C-VLL), Dr. Thomas Chused (NDR), Dr. H.C. Morse, III (NIAID)

Cooperating Units: NIAID, National Naval Medical Center, NCI-VLL, Georgetown University Hospital and D-OMS Branch.

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives:

New Zealand mice spontaneously develop an autoimmune disease resembling human systemic lupus erythematosus. Early in life they become relatively resistant to tolerance induction, later they spontaneously develop antibodies to RNA and DNA and finally they develop immune complex glomerulonephritis from which they die. Both humoral and cellular immune defects have been reported and are under active investigation.

Cellular cooperation between thymus derived (T) cells and bone marrow derived (B) cells is a recently described feature of humoral immunity. Two distinct T cell subpopulations cooperate in cell mediated immunity The existence of suppressor T cells has been suggested by the increased antibody response to a thymic independent antigen given with antithymocyte serum (ATS). We have been investigating such suppressor cell function in New Zealand mice in the hope of understanding the pathogenesis of their autoimmune disease.

Methods Employed:

1. Assay for antibodies to RNA and DNA using ^{14}C-labeled nucleic acids as ligands and the ammonium sulfate precipitation asssy. Assay of antibody forming cells to sheep erythrocytes and polysaccharide by local hemolysis in gel.

2. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants.

3. Transfer of spleen, bone marrow and thymus cells into recipients to evaluate the functional properties of the different cell types.

4. Use of standard skin grafting using allogenic skin to evaluate cellular immune functions in New Zealand mice, and transfer of serum and cell population between young and old New Zealand mice to study the effect in New Zealand mice and other mice.

5. Study of graft versus host disease inducing properties of NZB/W lymphoid cells using the Simonsen spleen weight assay and newborn C3H/He or SWISS mice as recipients.

6. Separation of lymphoid cells functionally (corticosteroid, ATS, anti-Θ, anti-kappa) and physically (sedimentation).

Major Findings:

1. Type III pneumococcal polysaccharide (SSSIII) was found not to cross react with nucleic acid antigens. The antibody response to immunization with this thymic independent antigen increased with age in NZB/W mice in contrast to a) a decrease with age in other mice, b) a decrease with age in NZB mice of the antibody response to sheep erythrocytes and other thymic dependent antigens.

The increased response of older NZB mice to SSSIII was reduced significantly by thymocytes from young NZB mice.

2. Suppressor cells have been studied in cellular immunity using the graft versus host reaction (GVH) model. The GVH response measured in newborn C3H mice to 5 x 10^6 NZB/W spleen cells increases between 1

and 6 months and then falls to low levels by 12 months. This age change does not occur in control mice. The high response of 4 1/2 to 6 month NZB/W spleen cells was suppressed by thymocytes or spleen cells from young NZB/W mice. Suppressive activity of thymus was lost gradually between 1 and 4 months of age. Thymocytes from 4 month NZB/W mice could synergize with 12 month NZB/W spleen cells, but could not suppress 4 1/2 month spleen cells, whereas thymocytes from 1 month NZB/W mice could synergize with 12 month NZB/W spleen cells and suppress 4 1/2 month spleen cells. Control strains showed neither suppression nor synergy.

3. Fractionation of spleen cells from young and old NZB/W mice and control strains show no gross differences in fractionation pattern. However, functional differences were observed. We were able to separate spleen cells that have high activity in the mixed lymphocyte reaction, and high responses to mitogens (PHA and Con A). Helper T cells in the response to SRBC were obtained in 2 fractions.

4. Three lymphoid cell populations were found to cooperate in skin allograft rejection in NZB/W and BALB/c mice. One T cell and one B cell subpopulation were present in old NZB/W mice. Missing in old NZB/W mice but present in young NZB/W mice was a recirculating T cell. We found that this was lost with age as a result of spontaneously occurring anti-T cell antibody. This antibody reproduced the defect in skin allograft rejection in young NZB/W mice.

5. Athymic "nude" mice were found to develop spontaneously antibodies to DNA and immune complex glomerulonephritis. These mice were breed on a BALB/c background and were known to be infected with several viruses. Athymic "nude" mice on a SWISS genetic background and not carrying these viruses did not develop autoimmunity.

Significance to Bio-Medical Research and the Program of the Institute:

These studies suggest that New Zealand mice lose thymic suppressor cells as they age leading to hyperactive antibody responses including autoantibodies, and the development of lymphoreticular malignancy. Later in life they lose helper cells leading to impaired cell mediated immunity. These studies suggest the possibility of similar pathogenetic mechanisms in humans.

Proposed Course:

Future studies will attempt to further define and separate different subpopulations of T cells, as well as the relative changes in such populations with age (helper, suppressor, effector, etc.). The role of genetic and viral factors in the loss of suppressor cells will be investigated in New Zealand mice. The availability of athymic nude mice will allow us to study lack of thymic function in different strains with and without superinfection with different viruses. We hope thereby to define the relative contributions of genetic, viral, and immunologic factors in the development of autoimmunity.

Honors and Awards: None

Publications:

1. Chused TM, Steinberg AD, Parker LM: Enhanced antibody response of mice to polyinosinic·polycytidylic acidy by anti-thymocyte serum and its age-dependent loss in NZB/W mice. J. Immunol. 111:52-57, 1973.

2. Hardin JA, Chused TM, Steinberg AD: Suppressor cells in the graft versus host reaction. J. Immunol. 111:650-651, 1973.

3. Gershwin ME, Steinberg AD: Loss of suppressor function as a cause of lymphoid malignancy. Lancet 2:1174-1176, 1973.

4. Barthold DR, Kysela S, Steinberg AD: Decline in suppressor T cell function with age in female NZB mice. J. Immunol. 112:9-16, 1974.

5. Parker LM, Chused TM, Steinberg AD: Immunofluorescent studies of the thymocytotoxic antibody of New Zealand mice. J. Immunol. 112: 285-292, 1974.

6. Steinberg AD: The pathogenesis of autoimmunity in New Zealand mice. V. Loss of thymic suppressor function. Arthritis Rheum. 17:11-14, 1974.

7. Gelfand MC, Steinberg AD: Cooperation of three lymphoid cells in the allograft rejection. Cell. Immunol. 11:221-230,1974.

8. Talal N, Steinberg AD: The pathogenesis of autoimmunity in New Zealand black mice. In Current Topics in Microbiology and Immunology, Springer-Verlag Publishers, 1974.

9. Gelfand MC, Parker LM, Steinberg AD: Mechanism of allograft rejection in New Zealand mice. II. Role of a serum factor. J. Immunol. , 1974.

10. Morse HC, III, Steinberg AD, Schur PH, Reed ND: Spontaneous "autoimmune disease" in nude mice. J. Immunol., in press.

Serial No. NIAMDD-ARB-22c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1. 1973 through June 30 , 1974

Project Title: Therapeutic studies in New Zealand mice

Previous Serial Number: 13C

Principal Investigator: Dr. Alfred D. Steinberg

Other Investigators: Dr. Naomi Lynn Gerber, Dr. Merrill Eric Gershwin
Dr. Michael C. Gelfand (NIAID)

Cooperating Units: NIAID

Man Years:

Total:	1-1/4
Professional:	3/4
Other:	1/2

Project Description:

Objectives:

New Zealand mice, especially the NZB/NZW F_1 spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. These mice are an excellent model for comparing different immunosuppressive drug programs. In addition, as the immunologic basis for the disease becomes better understood, they offer a model for studying specific immunotherapy.

Methods Employed:

1. Comparison of different immunosuppressive drug regimens in natural history of New Zealand mice - antibodies to DNA, proteinuria and survival.

2. Study of thymus grafting and thymic hormone in an attempt at restoring normal regulatory functions and improve the natural history of New Zealand mice.

Major Findings:

New Zealand mice responded to a number of immunosuppressive drug regimens when started at 5 months of age or later in life if the animals did not have significant proteinuria. When they had signif-cant proteinuria they did not respond favorably to the immunosuppressive regimens that were effective earlier in life. Chlorambucil, an alkylating agent like cyclophosphamide but without the latter's bladder toxicity appears in a preliminary study to be effective treatment of disease in NZB/W mice.

2. Intermittent (every 2 weeks) grafting of thymuses from young NZB/W mice to syngeneic NZB/W females starting at 1 month of age and continued until 9 months of age led to increased survival despite negative aspects of repeated thymus grafting demonstrated by grafting thymuses from cortisone pretreated NZB/W mice.

Significance to Bio-Medical Research and the Program of the Institute:

These studies are designed to provide background experience for clinical studies in which the immunosuppressive drugs may be given in a more effective manner to humans with SLE.

Continued study in mice may provide the background information necessary for immunological intervention in humans with SLE.

Proposed Course:

Treatment of New Zealand mice with thymic hormone and other attempts at immunologic reconstitution.

Honors and Awards: None

Publications:

1. Kysela S, Steinberg AD: Increased survival of NZB/W mice given multiple syngenic young thymus grafts. Clin. Immunol. Immunopath. 2:133-137, 1973.

Serial No. NIAMDD-ARB-23C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Receptors for IgE on Mast Cell Plasma Membranes

Previous Serial Number: 17c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Anthony Kulczycki, Jr.
Dr. Chaviva Isersky-Carter
Dr. Dennis Carson
Dr. Donald Buell

Cooperating Units: National Cancer Institute - Immunology Branch

Man Years:

Total:	1 1/2
Professional:	1 1/4
Other:	1/4

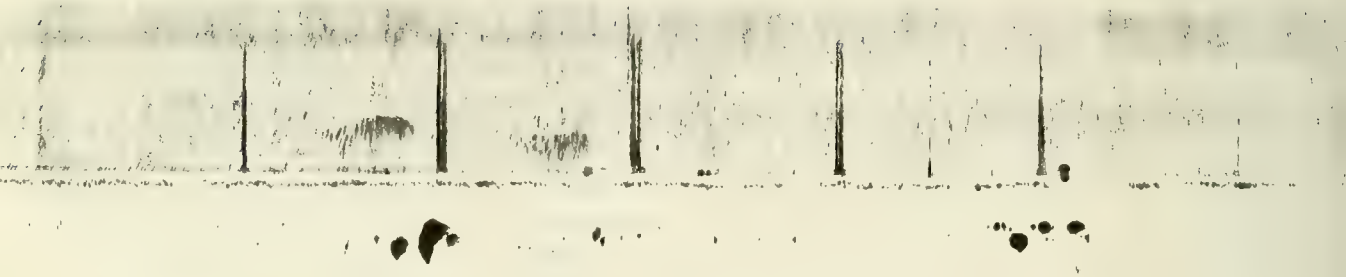
Project Description:

Objectives:

IgE is a unique class of immunoglobulin which plays a predominant role in the physiological events associated with a variety of allergic phenomena. The immunoglobulin becomes attached to the surface of basophils and mast cells and, upon exposure to the allergenic antigen, mediates the release of histamine and other vasoactive amines from the cells to which it is attached. Although there is an increasing amount of information concerning the biochemistry of this reaction almost nothing is known about how the IgE acts as the cell surface transducer. The present project is directed towards increasing our understanding of how the IgE interacts with mast cells.

Methods Employed:

Since our previous report we have made considerable progress in developing a system with which we can achieve our objectives. In March 1973 we received a litter of rats bearing a rat basophilic leukemia - a leukemia originally discovered by Dr. Brian Leonard (Imp. Chem. Industries, England; see Nature - New Biol. 244:73, 1973). We successfully transplanted this tumor to NIH Wistar-strain rats and have attempted to culture the cells and study their IgE binding properties using iodinated IgE (Serial No. ARB-24C ARB-26C 1973-74).



Serial No. NIAMDD-ARB-23C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Receptors for IgE on Mast Cell Plasma Membranes

Previous Serial Number: 17c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Anthony Kulczycki, Jr.
Dr. Chaviva Isersky-Carter
Dr. Dennis Carson
Dr. Donald Buell

Cooperating Units: National Cancer Institute - Immunology Branch

Man Years:

Total:	1 1/2
Professional:	1 1/4
Other:	1/4

Project Description:

Objectives:

IgE is a unique class of immunoglobulin which plays a predominant role in the physiological events associated with a variety of allergic phenomena. The immunoglobulin becomes attached to the surface of basophils and mast cells and, upon exposure to the allergenic antigen, mediates the release of histamine and other vasoactive amines from the cells to which it is attached. Although there is an increasing amount of information concerning the biochemistry of this reaction almost nothing is known about how the IgE acts as the cell surface transducer. The present project is directed towards increasing our understanding of how the IgE interacts with mast cells.

Methods Employed:

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Major Findings:

The following has been accomplished:

1. The leukemic cells have been adapted to suspension and spinner culture. The cells can be stored under liquid N_2 and revived.
2. The natural history of the cells during culture growth has been documented and the cell cycle defined.
3. It has been found that these cells contain histamine in approximately normal amounts.
4. The cells have been found to have a receptor for IgE which by all available criteria appears to be similar to the receptor found on normal (human) basophils and (rat) mast cells. {Unfortunately very little is known about "normal" receptors for IgE.}
5. The receptor properties have been rigorously studied with regard to:
   a. Number per cell (varies during cell cycle from 2.5×10^5 to 1.2×10^6)
   b. Mechanism of reaction (simple reversible binding)
   c. Forward rate constant (1×10^5 M^{-1} Sec^{-1})
   d. Reverse rate constant (1.4×10^{-5} Sec^{-1})
   e. Association constant (7×10^9 M^{-1})
   f. Temperature, pH, Ca^{++}, heparin, phosphatidyl serine, etc. dependence of binding
6. The 'natural history' of the receptor during the cell cycle has been studied (receptor activity increases during G1 but not in S_1, G_2 or M) During the lag phase after reinitiation growth from a stationary culture shedding of the receptor appears to occur.
7. The effect of IgE and anti-IgE concentration on redistributing the surface IgE (and its receptor) have been extensively investigated. These studies provide data which will be potentially useful in studying the properties of the receptor for IgE in situ and possibly for isolating the receptor.

Significance to Bio-Medical Research and the Program of the Institute:

It is of considerable importance to study the behavior of antibodies on the surface of cells in order to more completely understand the mechanism of the immune response. It is on the cell surface that most of the critical steps of the immune response transpire and it is here that one must direct one search for the ultimate mechanisms by which antigen-antibody reactions initiate specific cellular responses. We now have what we believe is the first instance of a system in which a receptor for an immunoglobulin can be studied rigorously. With it we can potentially make significant contributions to our understanding of antigen-induced, antibody-mediated, cellular reactions.

Proposed Course:

1. Isolation of membrane fraction with intact receptors.
2. Isolation characterization of soluble receptor.

3. Characterization of receptor organization in membrane.
4. Attempt to "trigger" cells to release histamine via an antibody-mediated reaction.

Honors and Awards: Dr. Kulczycki received the J. D. Lane Award of the Commissioned Officers Association of the U.S.P.H.S. in April 1974 for some of this work. It is made "for the most significant contribution in original research".

Publications:

Kulczycki, Jr. A, Isersky C, Metzger H: The interaction of IgE with rat basophilic leukemia cells. I. Evidence for specific binding of IgE. J Exp Med 139:600-616, 1974.

Serial No. NIAMDD-ARB-24C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Isolation of Rodent IgE

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Chaviva Isersky-Carter
Dr. Anthony Kulczycki, Jr.

Cooperating Units: None

Man Years:

Total:	1 1/2
Professional:	1 1/4
Other:	1/4

Project Description:

Objectives:

Although some of the properties of rat IgE had been previously characterized heretofore this immunoglobulin had not been isolated in a sufficiently purified form to be used for binding studies (Serial No. ARB- 23C 1973-74).

Methods Employed:

Routine physicochemical and immunochemical methods including immunoadsorbant techniques.

Major Findings:

Rat IgE antibodies were brought to a sufficient degree of purity to permit their use for cell binding studies. Approximate values for the IgE content in reaginic sera was determined and the amount of IgE required to obtain detectable passive cutaneous anaphylaxis reactions established.

Significance to Bio-Medical Research and the Program of the Institute:

1. This work has permitted us to develop a simple method for quantitating the IgE content of rodent sera (Serial No. ARB- 25C 1973-74). The IgE response in rodents has received considerable attention recently because it presents some unique problems in the regulation of the immune response.

2. We have developed what we believe to be a system uniquely suitable to study antibody-mediated cell activation. The preparation of labeled purified IgE has provided us with one of the vital components of this system.

Proposed Course:

This project has been completed.

Honors and Awards: None

Publications:

Isersky C, Kulczycki, Jr. A, Metzger H: Isolation of IgE from reaginic rich rat serum. J. Immunol. 1974 (In press)

Serial No. NIAMDD-ARB-25C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Quantitation of IgE

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Dennis Carson

Cooperating Units: Dr. Herve Bazin (University of Louvain-Belgium)

Man Years:

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Objectives:

To develop a simple method for quantitating IgE.

Methods Employed:

During the development of a method for purifying rat IgE (Serial No. ARB- 24C 1973-74) we discovered that rat IgE did not precipitate at $(NH_4)_2SO_4$ concentrations below 40% saturation. This is a property both of rat reaginic and myeloma IgE's. When rabbit anti-IgE becomes bound the IgE becomes precipitable. This has permitted us to develop a simple method for measuring IgE in biological fluids using a Farr type assay with radiolabelled IgE.

Major Findings:

The method has permitted us to establish IgE levels in newborn, adult unimmunized and immunized rats. The method can also be used for quantitating human IgE.

Significance to Bio-Medical Research and the Program of the Institute:

Many laboratories are interested in studying IgE immune responses in rodents. Previously only a cumbersome biological assay was available. The present method should be helpful in this regard.

Proposed Course:

Studies are planned to evaluate the role of parasitic injections in enhancing the IgE response to hapten-conjugates. In particular it is of interest to see whether such injections lead to a generalized increase in IgE levels apart from the specific anti-parasite IgE.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-26C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Structure of IgE in Relation to Biological Function

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. James Halper

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/4
Other:	1/4

Project Description:

Objectives:

To study structural/functional relationships in the IgE-basophil interaction.

Methods Employed:

Attempts will be made to isolate and characterize those portions of the IgE molecule which are involved in the binding to cell-receptors for IgE. A rat myeloma IgE and a rat basophil leukemia which has receptors for IgE (Serial No. ARB-23C 1973-74) will be used.

Major Findings: None to date

Significance to Bio-Medical Research and the Program of the Institute:

None

Proposed Course:

The structures on IgE which are important for IgE-basophil interactions are being investigated using a rat IgE myeloma.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-ARB-27C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunoglobulins on Cell Surfaces

Previous Serial Number: 16-c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Karl Becker, Dr. Philip M. Grimley, Dr. Teruko Ishizaka and Dr. Kimishige Ishizaka

Cooperating Units: Section on Pathological Anatomy, NCI and Departments of Medicine and Microbiology, Johns Hopkins University School of Medicine, Good Samaritan Hospital, Baltimore, Maryland.

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Proposed Course:

This project has been completed and led to a publication.

Publications:

Becker KE, Ishizaka T, Metzger H, Ishizaka K, Grimley P: Surface IgE on human basophils during histamine release. J Exp Med 138:394-409, 1973.

Serial No. NIAMDD-ARB-28C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on J-chain Content in Human (Waldenström) Macroglobulins

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Constantine Mihaesco
Dr. Edith Mihaesco

Cooperating Units: Laboratory of Immunochemistry
Res. Inst. of Blood Diseases
Hôpital St. Louis
Paris, France

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Objectives:

The role of J-chain in the structure of IgM remains uncertain; it may be important for the assembly of the molecule. It is important to know whether there is a rigid stoichiometry between J, light and heavy chains. Therefore it was decided to examine several Waldenström IgM molecules to see if the J chain content was the same in all of them.

Methods Employed:

A simple method for separating J chain from the other polypeptide chains was developed. Although J chain makes up as little as 1.5% of the total protein in an IgM molecule it has a relatively high number of easily reducible disulfide bonds. The method involves the use of radioactive iodoacetamide with which the reduced IgM is reacted and determining the ratio of counts in the J chain and non-J-chain peaks in a gel filtration experiment. By depending on the ratios rather than absolute measurements many factors which could influence the results were cancelled out. Other methods were also used for confirmatory data.

Major Findings:

The data were most consistent with each of the 6 IgM's examined having at least one J-chain per molecule but some of the proteins having as many as 2 moles of J-chain.

Significance to Bio-Medical Research and the Program of the Institute:

This study has added some information regarding the structure of an important class of immunoglobulins.

Proposed Course:

The project has been completed and led to a publication.

Honors and Awards: None

Publications:

Mihaesco C, Mihaesco E, Metzger H: Variable J-chain content in human IgM. FEBS Letters 37:303-306, 1973.

Serial No. NIAMDD-ARB-29C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Use of Divalent Haptens to Assess Antibody Structure

Previous Serial Number: None

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Dennis Carson

Cooperating Units: None

Man Years:

Total:	1
Professional	3/4
Other:	1/4

Project Description:

Objectives:

The laboratory of V. Schumaker has shown that some rabbit (IgG) anti-DNP antibody molecules are capable of forming dimers with divalent ligands while others are not. This unexpected finding could have important implications in regard to subclasses of rabbit IgG (which had never been observed) and to the intramolecular flexibility of IgG. We wished to find the explanantion of the phenomenon.

Methods Employed:

Reaction of divalent hapten analogues with intact IgG and its fragments.

Major Findings:

The monovalent fragments mimiced the behavior of the intact molecules. These results and others suggest that the degree of dimer formation is simply related to the depth of the antibody combining site.

Significance:

Our data provide further information on antibody structure particularly in relation to formation of antibody-antigen complexes.

Proposed Course:

No further studies planned.

Honors and Awards: None

Publications:

Carson D, Metzger H: The separation of rabbit antinitrophenyl IgG antibodies on the basis of combining site depth. Immunochem. 1974 (In press)

Serial No. NIAMDD-ARB-30C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on a Mouse Myeloma Protein having Antibody Activity

Previous Serial Number: 24c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Nortin M. Hadler

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Proposed Course:

This project has been completed and led to a publication.

Publications:

Hadler N, Metzger H: The modification of affinity labeled antibodies. Immunochem 10:455-460, 1973.

Serial No. NIAMDD-ARB-31C
1. Arthritis and Rheumatism
2. Chemical Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Combining Sites of Mouse Myeloma Proteins which Bind Phosphoryl Choline

Previous Serial Number: ARB-15c

Principal Investigator: Dr. Henry Metzger

Other Investigators: Dr. Bruce Chesebro, Dr. Nortin Hadler and Dr. Dennis Carson

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Proposed Course:

This project was discontinued because of technical difficulties encountered and changes of interest in the laboratory.

Publications:

Chesebro B, Hadler N, Metzger H: Affinity Labeling Studies on Five Mouse Myeloma Proteins which bind Phosphorylcholine. In Specific Receptors of Antibodies, Antigens and Cells. (D. Pressman, T. B. Tomasi, Jr., A. L. Grossberg, and N. R. Rose, eds.) pp. 205-217, S. Karger, Basel, Switzerland, 1973.

Serial No. NIAMDD-ARB-32c
1. Arthritis & Rheumatism
2. Human Biochemical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1,1973 through June 30, 1974

Project Title: The Biochemical Lesions in the Genetic Mucopolysaccharidoses and Mucolipidoses

Previous Serial Number: NIAMDD-LBM-10

Principal Investigator: Dr. Elizabeth Neufeld

Other Investigators: Dr. Scot Hickman, Dr. Larry Shapiro, Dr. Timple Lim, Mrs. Clara Hall, Mr. James Scott

Cooperating Units: Section on Intermediary Metabolism, LBM

Man Years:

Total:	6-1/4
Professional:	4
Others:	2-1/4

Project Description:

Objectives - To study the biochemical defect of genetic disorders of lysosome function, such as mucopolysaccharidoses and mucolipidoses and to use the findings for medical assistance to the patients and their families.

Methods Employed - Two enzymes, α-L-iduronidase and sulfoiduronate sulfatase are purified from human urine by a combination of previously described methods with affinity chromatography. The enzymatic activity, corrective factor activity, and capture by fibroblasts are determined. Cultured fibroblasts from patients, patients' relatives and normal individuals are used for measurement of enzyme levels, and patient's fibroblasts are used to determine uptake of enzyme.

Major Findings - 1. Purification of α-L-iduronidase from human urine has been simplified by the use of an affinity adsorbent (heparin-Sepharose). This has been done in collaboration with Dr. Irwin Leder, LBM.

2. An isozyme of iduronidase that acts as a corrective factor has been separated from an isozyme that does not. The functional difference between the two isozymes is in their ability to be taken up by fibroblasts. Iduronidase from patients with mucolipidoses II and III is not corrective.

3. An assay has been developed for sulfoiduronate sulfatase, based on the desulfation of a substrate labeled with ^{3}H, followed by chromatographic or electrophoretic separation of product and residual substrate.

Significance to Bio-medical Research and the Program of the Institute - Deficiency of the enzymes in question, α-L-iduronidase and sulfoiduronate sulfatase, results in the Hurler or Hunter syndrome, respectively. Our research has resulted in simple methods for diagnosis, prenatal as well as postnatal. The new assay for sulfoiduronate sulfatase may make it possible to detect the carriers among the female relatives of patients with the Hunter syndrome (an X-linked disorder). Purification of the enzymes and elucidation of how they are captured by the cells should make possible a clinical trial of enzyme replacement.

A previously unsuspected type of cell interaction has been revealed by studies of uptake of lysosomal enzymes into fibroblasts. In connective tissue, not only do cells exchange hydrolytic enzymes (i.e., one cell secreting and another capturing), but such interaction may be obligatory for packaging the hydrolases into lysosomes.

Proposed Course of Project - 1. Properties of the above-mentioned enzymes, such as molecular weight, amino acid composition and carbohydrate content will be examined as soon as the degree of purification allows meaningful measurements.

2. Antibodies will be prepared, and cell extracts from patients will be examined for cross-reactive proteins.

3. Differences will be sought between the isozymes (from normal individuals) that are captured by fibroblasts and those that are excluded; likewise, the properties of enzymes elaborated by patients with mucolipidoses II and III (I-cell disease and pseudoHurler polydystrophy, respectively) will be examined in order to explain the exclusion of these enzymes from fibroblasts (and, in the process, to pinpoint the primary defect in these genetic disorders).

4. The selective uptake of some lysosomal isozymes implies the existence of receptors for these enzymes on the fibroblast surface. Chemical evidence will be sought for such receptors. In mucolipidoses II and III hydrolases are defective but receptors are probably normal; conditions will be sought in which the situation is reversed.

5. Tissue and species specificity of enzyme capture will be examined.

6. Enzyme preparations will be examined for toxicity in anticipation of a clinical experiment within the next two years. Protocol will be developed for such an experiment.

Honors and Awards: National Lecturer of the Society of Biological Chemists (Minneapolis, June 3).

Publications:

Neufeld, E. F.: Replacement of genotype-specific proteins in mucopolysaccharidoses. In Desnick, R. J., Bernlohr, R. W., and Krivit, E. W. (Eds.): Enzyme Therapy in Genetic Diseases. Birth Defects. 9: 27-30, 1973.

Neufeld, E. F., Kresse, H. M., Hall, C. W., Cantz, M. J., and Barton, R. W.: The mucopolysaccharidoses: An illustration of genetic heterogeneity and prospects for therapy. In de Grouchy, J., Ebling, J. G., and Henderson, I. W. (Eds.): Proc. IV Int'l. Congr. Human Genetics, Paris 1971. Excerpte Medica, Amsterdam, 1972, pp. 31-35.

McKusick, V. A., Howell, R. R., Hussels, I. E., Neufeld, E. F., and Stevenson, R.: Allelism and genetic compounds among the mucopolysaccharidoses. Corrective factors in nosology, genetics and therapy. Trans. Assoc. Am. Phys. 15: 151-171, 1972.

Neufeld, E. F., and Cantz, M.: The mucopolysaccharidoses studied in cell culture. In Hers, H. G., and van Hoof, F. (Eds.): Lysosomes and Storage Diseases. New York, Academic Press, 1973, pp. 261-275.

Hall, C. W., and Neufeld, E. F.: α-L-Iduronidase activity in cultured skin fibroblasts and amniotic fluid cells. Arch. Biochem. Biophys. 158: 817-821, 1973.

Bach, G., Eisenberg, F., Jr., Cantz, M., and Neufeld, E. F.: The defect in the Hunter syndrome: Deficiency of sulfoiduronate sulfatase. Proc. Nat. Acad. Sci. USA. 70: 2134-2138, 1973.

Neufeld, E. F.: Mucopolysaccharidoses: The biochemical approach. In McKusick, V. A., and Claiborne, R. (Eds.): Medical Genetics. Hospital Practice, 1973, pp. 141-147.

Hickman, S., Shapiro, L. J., and Neufeld, E. F.: A recognition marker required for uptake of a lysosomal enzyme by cultured fibroblasts. Biochem. Biophys. Res. Commun. 57: 55-61, 1974.

Lim, T. W., Leder, I. G., Bach, G., and Neufeld, E. F.: An assay for sulfoiduronate sulfatase (Hunter corrective factor). Carbohydr. Res. (in press).

Stevenson, R. E., Howell, R. R., and Neufeld, E. F.: Mucopolysaccharidoses. H. F. Conn and R. B. Conn, Jr. (Eds.): Current Diagnosis. (in press).

Neufeld, E. F.: The biochemical basis of mucopolysaccharidoses and mucolipidoses. In Steinberg, A. G., and Bearn, A. G. (Eds.): Progress in Medical Genetics. Grune and Stratton. (in press).

Serial No. NIAMDD-ARB-33c
1. Arthritis and Rheumatism
2. Connective Tissue Disease
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Metabolic transformation of certain compounds that may be significant in human health and disease.

Previous Serial Number: ARB-26c

Principal Investigator: Dr. Peter Goldman

Other Investigators: Dr. Sheldon Milstien

Cooperating Units: None

Proposed Course:

This project has been completed and led to a publication.

Publication:

Milstien S, Goldman P: The role of the intestinal microflora in the metabolism of guanidinosuccinic acid. J Bacteriology 114:641-644, 1973

A means has been developed for purifying renal receptors for calcitonin and parathyroid hormone. A series of homogenization and density gradient steps allowed recovery of a fraction of renal membranes enriched with respect to adenylate cyclase, sodium-potassium ATPase and receptors for parathyroid hormone and calcitonin. The receptors were identified by determining binding of radioiodinated hormones to membranes as well as by assays for adenylate cyclase. Preparations of brush borders from renal tubules (kindly provided by Dr. David Neville) are not enriched with respect to hormone-stimulated adenyl cyclase. It is concluded that the plasma membrane fractions purified in our laboratory represent the basal portions of renal tubular cells. Iodinated calcitonin, synthetic of salmon type, binds specifically with high affinity to the membranes. The K_m for binding is approximately 10^{-10} M. It should be possible to develop this system into a highly sensitive radioreceptor assay for calcitonin. It has been proven that receptors for calcitonin in bone and kidney are virtually identical in affinity for a series of calcitonin analogs that vary in affinity over ∿ 1000-fold range. The affinity of the calcitonin analogs for receptor sites parallels closely the biological potency of the polypeptide *in vivo*. Similar receptors for calcitonin have been identified in lymphoid cells isolated from human or other species and grown in tissue culture. Again the same order of affinities was obtained as found for renal and skeletal receptors of rats.
[Drs. Marx, Heath and Aurbach]

β-adrenergic Receptor of the Avian Erythrocytes.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes. Interaction of β-adrenergic agonists with receptor in these cells causes a specific stimulation of sodium transport. This effect is mediated through the adenylate cyclase-cyclic 3',5'-AMP (CAMP) system and can be reproduced by adding exogenous CAMP. Stimulation of sodium transport by catecholamines is specifically inhibited by propranolol.

Binding of catecholamine to receptor was necessary but not sufficient for activation of adenylate cyclase. The dihydroxyphenyl function of the catecholamine molecule was required for binding as well as for activation of adenylate cyclase. The secondary alcohol function at the β-carbon, stereoconfiguration at the β-carbon, and an amine function, primary or secondary, at the α-carbon were essential for adenylate cyclase activation but not for specific binding. The β-adrenergic blocker, propranolol, was a potent inhibitor of adenylate cyclase activity but a weak inhibitor of binding. It was concluded that in addition to catechol-specific binding, a further interaction between plasma membrane and hormone is necessary for activation of adenylate cyclase. Thus further interaction appears to involve a site specific for the ethanolamine portion of the catecholamine molecule.

Purine nucleotides enhance catecholamine-stimulated adenylate cyclase activity in the erythrocyte membrane. A site separate from the catalytic site for ATP involved in regulating adenylate cyclase activity has been identified by determining binding of radioactive guanylyl imidodiphosphate (GMPPNP) to the erythrocyte membrane. The affinities of various purine nucleotides for the "GMPPNP site" parallels closely the apparent affinities of the nucleotides in enhancing isoproterenol-stimulated adenylate cyclase. [Drs. Spiegel, Bilezikian and Aurbach]

A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization rathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral and thence through the major veins to reach the neck where selective samples are taken from each inferior thyroid and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10 to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to peripheral results. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by nonparathyroid cancers.
[Drs. Spiegel, Mallette, Heath and Aurbach, NIAMDD, Dr. Doppman, Radiology Department, C.C., and Drs. Powell and Potts, Massachusetts General Hospital]

A metabolic study of the effects of space flight on various chemical elements, particularly those with special relevance to the musculoskeletal system, was carried out on the nine astronauts who participated in the three Skylab flights of 28, 60 and 84 days in 1973-74. The study required of the cooperating crewmen quite constant dietary intake, continuous 24-hour urine collections and total fecal collections for 21-31 days before each flight, throughout each flight and for 17-18 days post-flight.

Increases in urinary calcium during space flight and in-flight total calcium losses were closely similar in degree to those found in immobilization-bedrest. Similarity in pattern in urinary calcium rises and in total calcium losses to bedrest suggested that calcium losses would continue for a very long time. Significant losses of nitrogen and phosphorus occurred, associated with muscle tissue loss. Both mineral and muscle losses occurred despite vigorous exercise regimens in flight. It was concluded that unless protective measures can be developed, capable musculoskeletal function will be threatened in ultimately to be conducted (Mars) space flights of 1-1/2 to 3 years.
[G. Donald Whedon (NIAMDD), Leo Lutwak (UCLA School of Medicine and VA Hospital, Sepulveda, Calif.), Jeanne Reid (NIAMDD), Paul Rambaut, Michael Whittle, Malcolm Smith and Carolyn Leach (all at Biomedical Research Division, Johnson Space Center, NASA, Houston, Texas]

Serial No. NIAMDD-MDB-1 (c)
1. Metabolic Diseases Branch
2. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies in Bone Metabolism

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. Allen Spiegel, James Phang and G. Donald Whedon

Cooperating Units: Dr. Mones Berman, Mathematical Research Branch, NIAMDD
Metabolism Branch, NCI

Man Years:

Total:	1.00
Professional:	.25
Other:	.75

Project Description:

Objectives: 1) To investigate the factors influencing mineral storage and loss in demineralizing bone diseases. 2) To investigate the response of the parathyroid gland to rapid changes in blood calcium. 3) To study the influence of phosphate on resorption of bone mineral.

Methods Employed: Metabolic balance studies in patients with disorders of calcium metabolism, noting the effects on nitrogen, calcium, phosphate and magnesium balances of various dietary intakes of calcium, phosphate and magnesium. 2) Estimation of pool sizes, turnover rates, bone deposition rates, and absorption rates of calcium in patients by the oral and intravenous administration of tracer doses of ^{47}Ca.

Major Findings: Same as those reported 7/1/68-6/30/73.

Honors and Awards: None

Publications:

1. Whedon, G. D., and Cole, W.: Winning the war against childhood diabetes. Parents Magazine, November 1973.

2. Shapiro, J.R., Moore, W.T., Jorgensen, H., Reid, J., Epps, C., and Whedon, G. D.: Evaluation of diagnosis and therapy in osteoporosis. Arch. Intern. Med. In press.

Serial No. NIAMDD-MDB-2 (c)
1. Metabolic Diseases Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Parathyroid Hormone: Physiological Regulation of Secretion; Physical, Chemical, and Immunochemical Properties; Biochemistry; and Structure, and Mechanism of Action.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. S.J. Marx, A.M. Spiegel, D.A. Heath, L.E. Mallette, J.L. Doppman, B. Patten, W.K. Engel, J.T. Potts, Jr., H.T. Keutmann, G.W. Tregear, and H. Niall.

Cooperating Units: Radiology Department, C.C., Intramural Research and Medical Neurology Branch, NINDS, and Endocrine Unit, Massachusetts General Hospital

Man Years:

Total:	5.00
Professional:	1.75
Other:	3.25

Project Description:

Objectives: To prepare purified parathyroid hormone in pilot-plant quantities; to study chemical properties and structure of the hormone; to relate chemical properties and structure to biological activity and to study the mechanism of action of the hormone; to develop a clinically useful test for circulating parathyroid hormone; to study the physiological regulation of secretion of the hormone.

Methods Employed: Pure parathyroid hormone is prepared from phenol extracts of bovine parathyroid glands by solvent fractionation, gel filtration and chromatography on carboxymethylcellulose. Purity is assessed by disc gel electrophoresis, ultracentrifugation, immunodiffusion and immunoelectrophoresis, and recovery of the amino- and carboxyl-terminal amino acids. The hormone is assayed in vivo in rats, in vitro by measuring hormonal activation of adenylate cyclase, or by a highly sensitive radioimmunoassay. Specific chemical and enzymatic methods are used to cleave the hormone to smaller polypeptide fragments. The polypeptide derivatives are sequentially degraded with exopeptidases and the Edman reaction. Adenylate cyclase is measured by determining conversion of α-^{32}P-labeled

ATP to cyclic 3',5'-AMP. Endogenous concentration of cyclic 3',5'-AMP is determined by 1) converting the cyclic nucleotide to ATP and measuring the latter with a ^{32}P-ATP exchange reaction or 2) radioimmunoassay.

Major Findings: Mechanism of Action. Our earlier studies showed that the parathyroid status of animals is an important factor regulating excretion of cyclic AMP in the urine. This finding led to development of the thesis that cyclic AMP is involved in the mediation of parathyroid hormone action. Subsequent work showed that parathyroid hormone *in vitro* activates adenylate cyclase in the kidney as well as in bone, the two organs known to be physiological receptors for action of the hormone. Adenylate cyclase in the kidney is found in two different anatomic zones; adenylate cyclase in the cortex responds specifically to parathyroid hormone whereas that in the medulla responds to vasopressin. Further studies show that virtually all the adenylate cyclase detectable in kidney tissue is found within the renal tubules. This work establishes that the receptor for parathyroid hormone in kidney resides within the renal cortical tubule as had been suspected but not proven by indirect *in vivo* physiological experiments.

Chemistry of Parathyroid Hormone. Application of the "sequenator" method to analysis of parathyroid hormone has allowed deduction of the complete amino acid sequence of bovine parathyroid hormone. The molecule is an 84-amino-acid polypeptide with alanine as the amino-terminal residue and glutamine at the C-terminus. There are two phenylalanines (residues 7 and 34), two methionines (residues 8 and 18), one tryptophan (23) and one tyrosine (43). Previous work from this laboratory showed that the biologically important region of the molecule was located at the amino terminus within a polypeptide smaller than that demarcated by the first aspartate residue. Deduction of the sequence of the molecule allowed chemical synthesis of an amino-terminal biologically active fragment. The tetratriacontapeptide representing residues 1-34 in the hormonal molecule was synthesized by a solid-phase method.

After deblocking and purification the synthetic peptide was tested for biological and immunological activity. The product showed qualitatively all the biological properties, *in vivo* as well as *in vitro*, of the native molecule. Thus, the protean effects of the hormone on both renal and skeletal tissue can be accounted for by the same limited region of the molecule. It is also implied that the receptor sites for the hormone must be similar in bone and kidney.

Human Parathyroid Hormone. Parathyroid hormone was purified from human parathyroid adenomas. Earlier studies proved that the human hormone is different immunologically from the bovine hormone. Amino acid analyses of the hormone purified from each source indicated approximately eight amino acid substitutions in the human hormone. The amino acid sequence has been completed for the first 37 amino acids at the amino-terminal end of the molecule (Dr. Hugh Niall, MGH, Boston). Based on this analysis,

Dr. Geoffrey Tregear (MGH, Boston) synthesized the tetratriacontapeptide representing the amino terminus of the molecule. The product is fully as active in vitro as in vivo as the hormone isolated from human parathyroid adenomas (Drs. Potts, Niall, O'Riordan, Keutmann and Aurbach).

Efforts are continuing to collect large amounts of human parathyroid tissue from which further quantities of human hormone can be purified. With the highly sophisticated techniques now available for sequence analysis it is projected that if a further 1 to 2 mg of human parathyroid hormone can be isolated in pure form it will be possible to determine the amino acid sequence in the C-terminal region represented by the remainder of the molecule, residues 38-84.

A series of studies has been carried out utilizing selective venous catheterization and a radioimmunoassay for parathyroid hormone for localization rathyroid adenomas. A high degree of success has been obtained in localizing these tumors preoperatively. The catheter is passed through the femoral and thence through the major veins to reach the neck where selective samples are taken from each inferior thyroid and superior thyroid vein. Radioimmunoassays show that veins draining parathyroid adenomas frequently contain concentrations of hormone 10- to 20-fold or more greater than the concentration in the peripheral plasma. In cases with single adenomas, this concentration ratio is greatest selectively in one vein draining the adenoma. The contralateral vein shows concentrations equal to peripheral results. Patients with hyperplasia of all four glands show concentrations of hormone in the thyroid veins on each side greater than concentrations in peripheral plasma. Usually in these cases the concentration ratio is not as high as for single adenomas. This technique utilizing specific radioimmunoassays and selective catheterization should be applicable also to determining the source of hormone in ectopic hyperparathyroidism produced by nonparathyroid cancers.
[Drs. Spiegel, Mallette, Heath and Aurbach, NIAMDD, Dr. Doppman, Radiology Department, C.C., and Drs. Powell and Potts, Massachusetts General Hospital]

Neuromuscular Disease in Primary Hyperparathyroidism. Muscular weakness has been a recognized complication of primary hyperparathyroidism for many years. A prospective study with detailed clinical examination, needle electromyography, nerve conduction studies and histochemical evaluation of quadriceps muscle biopsies was carried out in 16 consecutive patients with primary hyperparathyroidism. Fourteen of the 16 patients were found weak on clinical neurological examination. The weakness was most marked in proximal muscles of the lower extremities and affected muscles were frequently atrophic. Nerve conduction velocity studies were normal. Electromyograms showed brief duration small amplitude motor unit potentials in some patients, and abnormal high amplitude long duration polyphasic potentials, suggesting reinnervation in others. Several patients also showed a decreased number of motor units under voluntary control, suggesting denervation. The major finding on muscle biopsy was atrophy of individual type I and type II muscle fibers, with type II fibers more frequently and more extensively involved. The atrophic fibers were frequently small and angular and

stained darkly with NADH-tetrazolium reductase or nonspecific esterase, findings which are specific for denervating disease. Most patients were significnatly better after surgical correction of their disease, although the length of time for resolution of the weakness varied. Further studies of neuromuscular function are underway in subjects with secondary hyperparathyroidism and other forms of metabolic bone disease.
[Drs. Patten, Prince and Engel, NINDS, and Drs. Bilezikian, Mallette and Aurbach, NIAMDD]

Significance to Biomedical Research and Program of the Institute: Same as for July 1968-June 1973.

Proposed Course of Project: Same as for July 1968-June 1973.

Honors and Awards: Gordon Wilson Lecturer, American Clinical and Climatological Association, 1973 (G.D.Aurbach, M.D.).

Publications:

1. Aurbach, G. D.: Parathyroid. In Beeson, P. B., and McDermott, W. (Eds.): Cecil-Loeb Textbook of Medicine, 14th Edition. Philadelphia, W. B. Saunders Co. (In press).

2. Aurbach, G. D.: Biosynthesis, secretion and mechanism of action of parathyroid hormone. Trans. Am. Clin. Climatol. Assoc. 85: 78, 1974.

3. Aurbach, G. D., Mallette, L. E., Patten, B. M., Heath, D. A., Doppman, J. L., and Bilezikian, J. P.: Hyperparathyroidism: recent studies. Ann. Intern. Med. 79: 566-581, 1973.

4. Aurbach, G. D., and Phang, J. M.: Vitamin D, parathyroid hormone, and calcitonin. In Mountcastle, V. B. (Ed.): Medical Physiology, 13th Edition, Volume II. Saint Louis, The C. V. Mosby Co., 1974, pp. 1655-1695.

5. Bilezikian, J. P., Doppman, J. L., Shimkin, P. M., Powell, D., Wells, S. A., Heath, D. A., Ketcham, A. S., Monchik, J., Mallette, L. E., Potts, J. T., Jr., and Aurbach, G. D.: Preoperative localization of abnormal parathyroid tissue: cumulative experience with venous sampling and arteriography. Am. J. Med. 55: 505-514, 1973.

6. Bilezikian, J. P., Pearson, K., Powell, D., Ketcham, A. S., Doppman, J. L., and Aurbach, G. D.: Selective venous catheterization of the neck: localization of a parathyroid adenoma drained anomalously of nonthyroidal veins. J. Clin. Endocr. 36: 826-829, 1973.

7. Heath, D. A., and Aurbach, G. D.: Ectopic production of parathyroid hormone: a prototype of nonendocrine hormone synthesis. In Kryston, L. J., and Shaw, R. A. (Eds.): Endocrinology and Diabetes. New York, Grune & Stratton. (In press)

8. Heath, D. A., Shimkin, P. M., and Wolfe, D. R.: The preoperative diagnosis of primary hyperparathyroidism by selective venous catheterization of neck veins in patients with known malignant disease. J. Clin. Endocr. 38: 618-621, 1974.

9. Mallette, L. E., Bilezikian, J. P., Ketcham, A. S., and Aurbach, G.D. Parathyroid carcinoma in familial hyperparathyroidism. Am. J. Med. (In press)

10. Mallette, L. E., Bilezikian, J. P., Heath, D. A., and Aurbach, G. D.: Primary hyperparathyroidism: clinical and biochemical features. Medicine 53: 127-146, 1974.

11. Niall, H. D., Sauer, R. T., Jacobs, J. W., Keutmann, H. T., Segre, G. V., O'Riordan, J. L. H., Aurbach, G. D., and Potts, J. T., Jr.: The amino-acid sequence of the amino-terminal 37 residues of human parathyroid hormone. Proc. Nat. Acad. Sci. USA 71: 384-388, 1974.

12. Patten, B. M., Bilezikian, J. P., Mallette, L. E., Prince, A., Engel, W. K., and Aurbach, G. D.: Neuromuscular disease in primary hyperparathyroidism. Ann. Intern. Med. 80: 182-193, 1974.

13. Shimkin, P. M., Feldman, M. G., and Heath, D. A.: Parathyroid dysfunction, parathyroid hormone assay, and the surgeon. Am. J. Surg. 126: 534-538, 1973.

Serial No. NIAMDD-MDB-3 (c)
1. Metabolic Diseases Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on the Chemical Nature and Mode of Action of Thyrocalcitonin.

Previous Serial Number: Same

Principal Investigators: Drs. S. A. Marx and G. D. Aurbach
Dr. David A. Heath,
Other Investigators: Drs. Donald Buell, David Rabin, and Jesse Roth

Cooperating Units: Clinical Endocrinology Branch, NIAMDD
Immunology Branch, NCI

Man Years:
Total: 1.50
Professional: .75
Other: .75

Project Description:

Objectives: To study the interaction of calcitonin with its specific receptor target organs.

Methods Employed: The interaction of calcitonin with its receptors has been evaluated by determining adenylate cyclase as well as binding of radioiodinated calcitonin to membranes isolated from renal tissue and to lymphoid cells isolated from human and other species or grown in tissue culture.

Major Findings: A purification procedure for kidney membranes has been developed. It has been shown that the calcitonin receptor from the kidney is concentrated at the corticomedullary junction. The membranes which bind calcitonin and contain calcitonin-sensitive adenylate cyclase show low specific activities for renal brush border enzymes, indicating that calcitonin receptors are localized at an anti-luminal aspect of renal cells or at a site that does not contain brush borders. Utilizing a series of calcitonin analogs, dose response curves were compared for receptors from kidney and bone. The dose response curves in the two tissues are virtually superimposable, suggesting that the calcitonin receptor in the two organs has a similar molecular structure.

Calcitonin receptors with similar characteristics have been identified on cultured human lymphoid cells. Human calcitonin, when compared to salmon calcitonin, competed weakly for the receptor on this human tissue.

Significance to Biomedical Research and Program of the Institute: The current investigations should provide further insight into the structure function relationship in calcitonin. Calcitonin is a small polypeptide hormone and therefore lends itself well to studies using synthetic peptide fragments. The system is also useful characterizing hormone receptors present in kidney, bone, and other tissues.

Proposed Course of Project: Further studies are in progress to characterize further the interaction of calcitonin with its tissue receptors. It also will be of interest to solubilize calcitonin receptors and further characterize them. Purified lymphoid cell types are being characterized with respect to the density of calcitonin receptors on their surface. The whole cell system also may be applied to evaluate the regulation of receptor metabolism *in vitro* and in human disease states.

Honors and Awards: None

Publications:

1. Marx, S. J., Keutmann, H.T., Glossman, H., Woodard, C., and Aurbach, G.D.: Renal receptors for calcitonin: binding and degradation of hormone. J. Biol. Chem. 248: 4797-4802, 1973.

2. Heersche, J.N.M., Marcus, R., and Aurbach, G. D.: Calcitonin and the formation of 3',5'-AMP in bone and kidney. Endocrinology 94: 241-247, 1974.

3. Marx, S. J., and Aurbach, G. D.: Adenylate cyclase from kidney and bone. In Hardman, J.G., and O'Malley, B.W. (Eds.): Methods in Enzymology, New York, Academic Press, Inc. In press.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies on Pseudohypoparathyroidism and Related Disorders

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. L. E. Mallette and A. M. Spiegel

Cooperating Units: None

Man Years:

Total:	1.00
Professional:	.75
Other:	.25

Project Description:

In 1942 Albright and his associates described the features of a new clinical syndrome "pseudohypoparathyroidism." Patients with this disorder differ from those with idiopathic hypoparathyroidism in that they have characteristic constitutional features and they do not respond to exogenous parathyroid extract. Albright proposed that this disorder was attributable to lack of end organ sensitivity to normally-secreted endogenous parathyroid hormone. Our findings that parathyroid hormone action is likely mediated through activation of adenyl cyclase, led us to test cases of pseudohypoparathyroidism by giving intravenous parathyroid hormone and measuring cyclic 3',5'-AMP in the urine. Pseudopseudo-hypoparathyroidism is a variant of the disorder and may occur in the same families with pseudohypoparathyroidism. Pseudopseudohypoparathyroidism is characterized by similar constitutional features but no clinical evidence of hypoparathyroidism. Several other syndromes including Gardner's syndrome, basal cell nevus syndrome, syndrome of calcification of basal ganglia, and vitamin D-resistant osteomalacia have also been reported in the past as resistant to the phosphaturic action of parathyroid hormone. Current studies have been extended to include testing patients in these categories as well.

Methods: Parathyroid hormone purified in our laboratories was sterile-filtered for clinical use through courtesy of the N.I.H. Pharmacy. Radio-immunoassay of parathyroid hormone was carried out as described previously. Measurement of 3',5'-AMP was performed according to the procedure devised in this laboratory.

Major Findings: Approximately cases of this disorder have been described in the literature; we have examined of these to date. In normal subjects, parathyroid hormone causes a 30- to 60-fold rise in urinary excretion of cyclic AMP within 30 minutes or less after intravenous administration of the hormone. A similar response has been observed in idiopathic, hypoparathyroid, surgical hypoparathyroid, and pseudopseudohypoparathyroid subjects (the latter category show the constitutional changes but do not have laboratory evidence of hypoparathyroidism). Parathyroid hormone caused little or no increase in excretion of cyclic AMP in patients with pseudohypoparathyroidism. In one case, studied through the cooperation of a clinical investigator at another hospital, there was a moderate rise in excretion of cyclic AMP; however, the diagnosis in this instance may be incorrect, and as yet we have not had the opportunity of seeing the patient at the Clinical Center. Patients with this disorder who fail to respond to parathyroid hormone showed a similarly defective response after prolonged calcium infusion. Parathyroid hormone was detectable in the plasma of pseudohypoparathyroid subjects before infusion of calcium but not after. This experiment tended to rule out the possibility that an abnormal endogenously secreted hormone immunologically reactive but biologically inert interfered with the action of exogenous parathyroid hormone.

The best evidence to date is that pseudohypoparathyroidism represents a genetic defect inherited as a sex-linked, dominant trait. There have been no documented cases of direct male-to-male transmission of the disease and the sex incidence is approximately 2:1 female-to-male. Several pedigrees have been described wherein pseudophyoparathyroid subjects were progeny of pseudopseudohypoparathyroid parents or vice versa. One family studied at the N.I.H. included a mother with pseudopseudohypoparathyroidism and her daughter with pseudohypoparathyroidism. The daughter failed to respond to exogenous hormone but the mother responded normally. We have also observed that, although the response to pseudopseudohypoparathyroidism is normal to exogenous hormone, there is a significantly increased rate of baseline excretion of cyclic AMP in this group. This observation may be of importance in further delineation of the precise genetic mechanism involved in transmission of the disorder. It appears likely that the cause of the disorder may be the existence of genetically deficient or defective adenylate cyclase in the bone and kidney of these subjects.

There is an association of hypothyroidism with pseudohypoparathyroidism and in a few such cases the hypothyroidism has been attributed to selective deficiency of thyrotropin. It seems possible that hypothyroidism in pseudohypoparathyroidism might reflect an abnormal receptor adenylate cyclase complex in the central nervous system or anterior pituitary analogous to the defect in the receptor for parathyroid hormone in kidney and bone. Three patients with coexistent hypothyroidism and pseudohypoparathyroidism were given thyrotropin-releasing hormone as part of a study designed to localize the site of the thyroid defect. One case showed an abnormally low basal concentration of thyrotropin in plasma, but gave a normal response to thyrotropin-releasing hormone. In the two other cases, a mother and

daughter, there were high basal concentrations of thyrotropin in plasma. The mother was tested with TRH and showed the exaggerated response to TSH secretion characteristic of primary hypothyroidism. The defect in the first case appears to be at the level of the hypothalamus or higher. The defect in the second two cases may represent an abnormality of the thyroid receptor for TSH itself.

Patients with Cushing's syndrome, basal cell nevous syndrome, syndrome of calcification of the basal ganglia, and vitamin D-resistant osteomalacia showed normal responses to parathyroid hormone as determined by urinary excretion of 3',5'-AMP. It was concluded that the classical phosphaturic response to parathyroid hormone is neither precise nor sensitive enough to discriminate normal subjects from those refractory to parathyroid hormone.

Kidney tissue was obtained from a patient with pseudohypoparathyroidism who died suddenly with complications of a massive pulmonary embolus. It was demonstrated in vitro that the membrane fraction of this kidney contained adenylate cyclase activity which was stimulatable by parathyroid hormone. Thus, it was concluded that pseudohypoparathyroidism did not represent a total lack of the renal parathyroid hormone-sensitive adenylate cyclase system.

Significance to Biomedical Research and Program of the Institute: Same as reported for July 1, 1968-June 30, 1973.

Proposed Course of Project: Same as reported for July 1, 1968-June 30, 1973.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-MDB-5 (c)
1. Metabolic Diseases Branch
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Hormone-Mediated Solute Transport: Interaction of Hormones with Cell Receptors; Interrelationship between Biogeneration of Cyclic AMP and Active or Facilitated Transport.

Previous Serial Number: Same

Principal Investigator: Dr. G. D. Aurbach

Other Investigators: Drs. A. M. Spiegel, J. Gardner and H. Klaeveman

Cooperating Units: Section on Gastroenterology, Digestive Diseases Branch, NIAMDD

Man Years:

Total:	1.75
Professional:	.75
Other:	1.00

Project Description:

Objectives: To isolate and purify specific hormone receptors in particular receptor tissues; to map the function of the receptor against activation of adenylate cyclase; to decipher the putative link between generation of cyclic 3',5'-AMP and solute transport in particular cell systems.

Methods Employed: Polypeptide and catecholamine hormones are purified and iodinated by standard methods or methods developed in the laboratory specifically for that purpose. Rate of association of hormones is determined by incubating plasma membrane fractions or whole cell with labeled hormones. The kinetics of binding is determined by rate measurements as well as assessment of steady state conditions. Standard mathematical analyses of results are performed utilizing double reciprocal or Scatchard plots. Specific hormone binding is defined in terms of dissociation constant (K_m) of the system. Physiologically significant binding shows K_m's compatible with the dose-response relationship recognized for the hormone at the physiological level.

The interaction of catecholamines with the β-adrenergic receptor and activation of adenylate cyclase were studied with the plasma membrane of turkey erythrocytes. Interaction of β-adrenergic agonists with receptor in these cells causes a specific stimulation of sodium transport. This effect is mediated through the adenylate cyclase-cyclic 3',5'-AMP (CAMP) system and can be reproduced by adding exogenous CAMP. Stimulation of sodium transport by catecholamines is specifically inhibited by propranolol.

The dihydroxyphenyl function of the catecholamine molecule was required for binding as well as for activation of adenylate cyclase. The secondary alcohol function at the β-carbon, stereoconfiguration at the β-carbon, and an amine function, primary or secondary, at the α-carbon were essential for adenylate cyclase activation but not for specific binding. The β-adrenergic blocker, propranolol, was a potent inhibitor of adenylate cyclase activity but a weak inhibitor of binding. It was concluded that in addition to catechol-specific binding, a further interaction between plasma membrane and hormone is necessary for activation of adenylate cyclase. Thus further interaction appears to involve a site specific for the ethanolamine portion of the catecholamine molecule.

Purine nucleotides enhance catecholamine-stimulated adenylate cyclase activity in the erythrocyte membrane. 5'-Guanylyl imidodiphosphate (GMPPNP), an analog of GTP, augments isoproterenol-stimulated adenylate cyclase causing greater activation of the enzyme than with 7 mM sodium fluoride. The effect occurs with a K_m for GMPPNP of 10^{-7} M. A similar K_m was observed for the slight stimulation of the enzyme by the nucleotide in the absence of added hormone. The greatly enhanced stimulation of adenylate cyclase activity by isoproterenol in the presence of GMPPNP is blocked completely by propranolol. The latter does not, however, abolish the stimulation of basal activity produced by the nucleotide. The K_m for isoproterenol activation of adenylate cyclase is decreased one order of magnitude by GMPPNP. GMPPNP binds to the cell membranes with a K_m equivalent to that found for effects on adenylate cyclase activity. Propranolol does not inhibit binding of the nucleotide, but unlabeled GMPPNP and other nucleotides competitively displace labeled GMPPNP from its binding sites with apparent affinities comparable to the order of potencies for augmenting catecholamine-stimulated adenylate cyclase. Displacement of GMPPNP from the binding site by GTP or ITP (GTP 100 X as effective as ITP) decreases enzyme activity activated by isoproterenol in the presence of GMPPNP. Thus, a high affinity purine nucleotide site is involved in regulating the catalytic function of the adenylate cyclase complex. The apparent affinity of the site for ATP is much lower than for GTP or GMPPNP. This finding indicates that, although ATP is clearly the substrate for the enzyme, it is GTP and not ATP that interacts at the regulatory site (identified as a GMPPNP binding site).

Significance to Biomedical Research and Program of the Institute:
There are a number of disease states which probably represent abnormalities of hormone receptors or the coupling between receptor, generation of cyclic AMP and physiological response to the hormone. This project represents the possible development of a model system wherein the connection between receptor binding of hormone, generation of cyclic AMP and transport can be investigated. Diseases such as nephrogenic diabetes insipidus or pseudohypoparathyroidism can better be explained once the inter-connecting links between these cellular events is understood.

Proposed Course of Project: Continuing studies are directed towards isolation of the receptors for particular hormones in receptor cells; solubilization and purification of the receptor; analysis of the inter-connecting substance between binding and adenylate cyclase activation; isolation of the guanyl nucleotide binding protein; identification of the system activated by cyclic AMP in stimulating transport of solute.

Honors and Awards: None

Publications:

1. Bilezikian, J. P., and Aurbach, G. D.: A beta-adrenergic receptor of the turkey erythrocyte. I. Binding of catecholamine and relationship to adenylate cyclase activity. J. Biol. Chem. 248: 5577-5583, 1973.

2. Bilezikian, J. P., and Aurbach, G. D.: A beta-adrenergic receptor of the avian turkey erythrocyte. II. Characterization and solubilization of the receptor. J. Biol. Chem. 248: 5584-5589, 1973.

3. Bilezikian, J. P., and Aurbach, G. D.: The effects of nucleotides on the expression of beta-adrenergic adenylate cyclase activity in membranes from turkey erythrocytes. J. Biol. Chem. 249: 151-161, 1974.

DIGESTIVE DISEASES BRANCH, NIAMDD

At the beginning of FY 1974, Dr. Leonard Laster, Chief of the Digestive and Hereditary Diseases Branch, retired from the Institute. Dr. Laster had been Chief of Gastroenterology (a section in the Metabolic Diseases Branch until 1969) since its original inception in 1965, and had made major contributions toward its growth, not by his own personal investigative work, but by interesting other capable investigators, both within and outside of NIAMDD, in research on disorders of the digestive system and liver.

In August 1973 the DHDB was renamed the Digestive Diseases Branch; and Dr. Robert Gordon, Clinical Director and former Chief of the Metabolic Diseases Branch, was named to head the expanded branch. Two sections formerly part of MDB, Phoenix Clinical Research Section and the Section on Physiology and Clinical Nutrition, were incorporated into the new DDB, along with Dr. Laster's former group, now designated as the Section on Gastroenterology, with Dr. Jerry Gardner as Chief, and the Section on Diseases of the Liver, under Dr. Paul Berk. The Branch, as presently constituted, has wide-ranging research interests, involving mechanisms of secretion and absorption in the gut and other model tissues, transport processes in the liver, immunologic aspects of diseases of the small bowel, cholesterol metabolism and transport as related to gallstone disease, and clinical trials of methodologies for the nonsurgical treatment of gallstones. Other clinical studies, initiated very recently, deal with improvement of methods for preparing patients for diagnostic study of the colon by X-ray and endoscopy, and with the natural history of vinyl-chloride-induced liver disease and hemangiosarcoma.

FY 1974 also witnessed events that promise even greater capabilities for the future. Dr. Berk and Dr. Gardner have each recruited a second qualified investigator, Drs. Anthony Jones and Kevin McCarthy, respectively. These two scientists will begin work in the coming fiscal year, and their clinical and laboratory skills and interests will supplement those of the section chiefs. A broader coverage of teaching and consultative activities will now be possible, and consideration will be given to the development of interplay, particularly between the sections on Diseases of the Liver and Gastroenterology, in providing for elective teaching for medical students, and for extending the clinical experience of Associates, insofar as the latter may wish it and specialty boards require it.

In January 1974, Dr. Gordon assumed the position of Director, Clinical Center, and Associate Director for Clinical Care, NIH. This move necessitated his relinquishing the Clinical Directorship of NIAMDD, but he will continue his interest in clinical research on diseases of the alimentary tract, and will serve as Chief of the Digestive Diseases Branch. Those laboratory projects not directly related to digestive diseases, of which he had been principal investigator (total energy metabolism and secretory mechanisms in sweat glands), are to be phased out.

Section on Physiology & Clinical Nutrition, DDB

FY 1974 will probably prove to be a terminal year for this section, since its chief and principal investigator, Dr. Robert Gordon, has become Director of the Clinical Center, and Associate Director for Clinical Care, NIH, beginning in January 1974. The year was to a large extent, therefore, devoted to the conclusion of studies which had been underway.

Progress has been made in the studies of mechanisms of secretion in human eccrine sweat glands. In an *in vivo* study using normal volunteers, Drs. Benson, Gordon, and Thompson showed that even after a week of starvation, a thermal stimulus would cause these glands to produce sweat containing almost as much lactate as was found with normal feeding; it thus appears that this tissue does not adapt to starvation by basing its energy metabolisms on fat, as does most of the body. With the help of Dr. Buja, NHLI, they demonstrated the surprising fact that sweat gland glycogen, depleted by thermal sweating, was replenished within a few hours even after a week of total starvation. Dr. Benson and Mrs. Chu are currently working with surviving sweat glands *in vitro*, and hope to be able to show whether or not this tissue is affected by insulin, with respect either to its ability to make lactate from glucose, or to its uptake of glucose from the medium.

Another investigation of sweating is in the final stages of data analysis prior to publication. Physiologists have wondered for years how human eccrine sweat comes to contain urea in a concentration higher than that of blood; it now appears on the basis of *in vivo* isotope studies that there exists within human skin a pool of urea that is not freely exchangeable with blood urea. This pool appears to be located in the epidermis, and is probably concentrated by simple evaporation of water to the atmosphere. In collaboration with Dr. Mones Berman, NCI, Dr. Gordon has constructed a mathematical model on the basis of these physiologic findings which fits the isotope data excellently.

With Dr. Gordon's change of responsibilities and Dr. Thompson's probable retirement impending, the section has decided not to press forward with the reinstrumentation of the metabolic chamber for energy balance studies. The chamber has served admirably as a controlled temperature room for the above-mentioned sweat studies, and for investigation of disturbances of thermo-regulation in a number of miscellaneous conditions, primarily using patients referred by other Institutes. Probably the most interesting of these is the situation in anorexia nervosa, where a consistent and reproducible defect in temperature regulation has been found in what many had considered to be a purely emotional disorder. The facts that the defect persists, even after remission of the anorexia and restoration of normal or near-normal body weight in anorectics, and that it cannot be demonstrated in thin persons who lack the other clinical features of anorexia nervosa, suggest that there is at least dysfunction at a sub-cortical level of the brain in these patients.

During 1974 two clinical studies have been initiated, with Dr. Gordon as principal investigator, that will be carried forward. One deals with the use of lavage of the entire GI tract, by means of saline solutions ingested voluntarily, as a means of preparing patients for barium enema and other X-ray studies, and for proctoscopy, colonoscopy, or other endoscopic procedures. Drinking saline may prove to be more acceptable to patients than the traditional procedures using castor oil, or other purgatives, followed by multiple enemas, and may have less irritant effect on the colonic mucosa, thus improving accuracy of the diagnostic studies. If this proves to be the case, both applicability and accuracy of these tests in the study of any disease affecting the colon may be improved. The other concerns itself with a prospective study of the possibility (admittedly not a high probability) that chronic administration of phenobarbital, by its inducing effect on hepatic microsomal enzyme systems, may so increase endogenous production of bile acids that cholesterol gallstone disease may be relieved. It does not seem likely that this study will have a positive result, but the disquieting effects of bile acid feeding on liver chemistries, and the cost and other side effects of the use of chenodeoxycholic acid are such that it was felt worth while to rule out the possibility that phenobarbital alone might be effective.

Section on Diseases of the Liver

Paul D. Berk, M.D., Chief

Since the Section on Diseases of the Liver is approaching the end of its first year of operation, the following brief outline of our activities may be of interest.

I. Principal Clinical Study

A) Studies of hepatic organic anion transport in man: development of techniques for quantitating individual steps in the hepatic transport of various cholephilic anions, by kinetic studies with BSP, indocyanine green, cholylglycine and radiolabeled bilirubin. In the past year kinetic studies in approximately 15 patients with Gilbert's syndrome have clearly demonstrated that this condition represents a heterogeneous population with at least three different sets of kinetic defects, some of which are clearly not attributable to defects in glucuronide conjugation.

II. Principal Non-clinical Study

A) Evaluation of albumin-agarose affinity chromatography hemoperfusion for the management of neonatal jaundice: studies in premature newborn sheep and Rhesus monkeys (in collaboration with Dr. Paul Plotz and with Dr. Ronald Chez, NICHHD).

III. Additional Projects in Preliminary Stages

A) Quantitative determination of heme turnover, and of the origin of bile pigments: validation of measurements of carbon monoxide production on plasma bilirubin turnover as accurate indicators of heme catabolic rate. Determination of porphyrins and bile pigments, based on the kinetics of labeling of various pigments isolated from blood, urine and feces after simultaneous intravenous administration of bilirubin-^{14}C, glycine-^{15}N, and δ-aminolevulinic acid-2,3-^{3}H (in collaboration with Dr. Samuel Schwartz, University of Minn.). Studies of the effects of various perturbations such as drug therapy or starvation on heme turnover.

B) Studies of radiocopper kinetics in Wilson's disease: goal is development of a model of copper metabolism the parameters of which will permit genetic differentiation among asymptomatic relatives of affected patients (i.e. presymptomatic disease vs carrier heterozygote vs normal) (in collaboration with Dr. Sean O'Reilly, George Washington University).

C) Characterization of the nature of pre-malignant liver disease in vinyl chloride workers: project seeks to find an effective screening protocol,

and to characterize the pathophysiology, mechanism and natural history of vinyl chloride induced hepatic fibrosis. At our request, patients with vinyl chloride induced angiosarcoma are being admitted to the Medicine Branch, NCI, for chemotherapy, and are also accessible for study.

D) Evaluation of the mechanisms of hepatic failure following bone marrow transplantation: hepatic failure, with an acute Budd-Chiari-like clinical picture, has been a major cause of death in the NCI marrow transplant program. To date, detailed review of liver obtained at autopsy from 11 of 30 available cases has detected a unique, and previously unrecognized proliferative lesion of the small hepatic veins, resulting in outflow block. We are currently attempting to correlate this lesion, both retrospectively and prospectively, with other evidence of graft versus host disease, and with preparative radio- and chemotherapeutic regimes in order to determine the pathogenetic mechanism (in collaboration with Dr. Hans Popper).

E) Natural history of chronic active liver disease detected on routine screening in the asymptomatic subject (in collaboration with Dr. Paul Plotz and Dr. Leonard Seeff, Washington V. A. Hospital). A long-term longitudinal study of an increasingly common problem afflicting, among others, at least five members of the NIH staff.

F) Studies of the adsorptive properties and biocompatibility of various synthetic resins which are potential candidates for inclusion in an artificial hepatic support system. As part of this project, various chemical and surgical animal models of hepatic failure are being studied as potential tools for the evaluation of such a system.

G) Isolation and purification of rat liver organic anion binding protein (Ligandin, Y Protein) by affinity chromatography. We hope soon to proceed to similar purification of the corresponding human protein in collaboration with Dr. William Jakoby.

H) Evaluation of changes in the nature of bilirubin conjugates produced in different cholestatic liver diseases: application of a high resolution thin layer chromatographic technique to plasma samples from patients with cholestatic conditions.

IV. Consultation Service

Consult load has increased progressively since July, and projects at an annual average of two consults and one biopsy per week. Bulk of requests are from NCI, which is now recognizing a very high evidence of previously uncharacterized liver disease in patients on chemotherapy, and from NIAID. We are attempting to set up a prospective study of the NCI problem, employing the services of Dr. Kamal Ishak, AFIP, to assess role of electron microscopy in distinguishing viral from drug-induced hepatitis. Consult service has required two-thirds of the time of one Clinical Associate, and we have arranged to have second year G.I. fellows from the V. A. Hospital rotate with us on three month electives to assist with the load. The initial V. A. fellow, Dr. Ghulan Rafat, is at NIH now, and is performing extremely well. As further help with the consultation service, an Attending Physician rotation involving

four NIH staff people, and eight clinical hepatologists and two pathologists from other Washington area institutions has been established. Attendings make rounds twice weekly to assist with both patient care and clinical teaching. Clinical Associates also attend weekly liver biopsy review conference at AFIP. On the basis of the formal teaching program established, we will shortly apply to appropriate Board Certification organizations to assure that Clinical Associates obtain two years' credit toward Board Certification in Internal Medicine and Gastroenterology. This may be of considerable help in future recruiting.

The arrival of Dr. Anthony Jones in July should accelerate progress in several of the clinical projects and add immeasurably to our clinical capabilities. In addition, Dr. Jones is planning to establish new projects in the areas of hepatic protein biosynthesis, and the immunology of the liver lesion associated with chronic inflammatory bowel disease (in collaboration with Dr. Warren Strober, NCI).

Section on Gastroenterology, DDB

The work of this section is directed toward understanding the origin and treatment of diseases involving the gastrointestinal tract by examining derangements of underlying processes involving cellular metabolism and physiology.

Immunophysiology of the human small intestine

During this past year we have applied our techniques for measuring immunoglobulin synthesis by the human small intestine from patients with gluten-sensitive enteropathy (GSE) to studies of the pathogenesis of ulcerative ileojejunitis. Our results indicate that patients with this disorder initially respond to dietary gluten exactly like patients with GSE, but as their disease progresses, develop a persistently elevated synthesis of IgA and damage to the small intestinal mucosa which (unlike patients with GSE) does not improve following withdrawal of dietary gluten. Results from studies of additional patients with GSE indicate that determination of small bowel immunoglobulin synthesis is a sensitive, reliable diagnostic test for sprue. (H. L. Klaeveman, A. G. Levy, C. S. Sessoms, W. Strober, A. J. Tousimis and J. D. Gardner)

Studies of membrane function

During this past year we have solubilized and begun to isolate the plasma membrane receptor for cardioactive glycosides. The functional characteristics of the receptor after solubilization differ significantly from those of the receptor in the intact cell. These differences when studied in conjunction with corresponding changes in chemical composition of the membrane should provide insight into the mechanism by which monovalent cations cross cell membranes as well as how it is perturbed by cardioactive glycosides. (J. D. Gardner, H. L. Klaeveman, A. G. Levy, C. S. Sessoms, J. R. Gill, Jr. and V. P. Butler, Jr.)

Gastrointestinal hormones

During this past year a major effort has been made toward elucidating the mechanism of action of cholecystokinin, secretin and vasoactive intestinal polypeptide. We have developed several systems for studying the interaction of these hormones with plasma membranes prepared from a homogeneous suspension of isolated cells. To date we have been unable to accomplish this for gastrin, and during the next year a major effort will be made to develop a system with which we can study the medium of action of gastrin. (J. D. Gardner, H. L. Klaeveman, T. D. Adams and T. P. Conlon)

Cyclic nucleotide-mediated alteration of cell function

During this past year we have explored further the mechanisms through which cyclic AMP stimulates membrane transport of sodium and potassium in avian erythrocytes and transport of amino acids in isolated thymic lymphocytes. These studies make it clear that cyclic nucleotides play an essential

role in regulating the chemical composition of the intracellular fluid and by so doing, mediate many of the diverse biologic effects of polypeptide hormones, β-adrenergic catecholamines and cholera toxin. (J. D. Gardner, H. L. Klaeveman, A. G. Levy, G. D. Aurbach, T. P. Conlon, T. D. Adams and A. J. Tousimis)

Gastric emptying

We have initiated a project designed to characterize the relevant physiologic variables which regulate gastric emptying. Thus far we have found that emptying of gastric contents is not a simple exponential function of the gastric volume but, instead, is a complex multicomponent process which is influenced by the volume and chemical composition of the gastric contents. (J. D. Gardner, A. Dubois, M. Berman and C. S. Sessoms)

Investigations to further knowledge of the physiology of bile formation and secretion, the pathophysiology of cholelithiasis, and therapeutic approaches to the dissolution of cholesterol gallstones have been conducted during the past year.

A simplified isotope dilution technique to measure total bile acid pool size requiring only a 12 hour period following isotope administratic has been developed. The method was shown to be precise and reproducible, thus providing a very useful tool for the investigation of the physiology of biliary secretion, and making feasible measurements of bile acid pool size in large numbers of subjects. This method would make possible, for example the screening of subjects with a high risk of cholelithiasis to determine if a reduced bile acid pool size is a primary lesion leading to cholelithiasis, or if the reduction in bile acid pool size found in cholelithiasis is secondary to its presence. (Drs. Bennett, Duane, Bennion and Ginsberg)

The differential cholesterol solubilizing capabilities of cholic and chenodeoxycholic acid derivatives have been determined by studies of the critical micellar concentration of the bile acid-lecithin mixed micelle.

The ability of the taurine and glycine conjugates of chenodeoxycholic acid to form micelles, and thus to solubilize cholesterol, was found to be much greater than with the same cholic acid conjugates.

These findings have enhanced our understanding of the physical chemical properties of bile and may well, in part, explain the differences in the capability of chenodeoxycholic and cholic acid to dissolve cholesterol gallstones in man. (Drs. Bennett and Duane)

Previous work in this laboratory showed that the lithogenicity of bile varied during the day. In an attempt to identify the mechanism for this variation, bile acid synthesis and pool size and the lipid composition of bile has been studied in the fed state and following fasts of four days. Little change in bile acid pool size was found, but cholic acid synthesis and fractional catabolic rates were consistently reduced. The molar percent cholesterol also diminished in the short-term fasted state. These results indicate that the diurnal variation in biliary lithogenicity cannot be simply explained on the basis of overnight fasting. (Drs. Bennett, Duane, Ginsberg and Bennion)

Since gallstones are more frequent in the obese and as cholesterol synthesis is higher in obesity it has been postulated that gallstone formation may be the result of obesity. To investigate this hypothesis biliary lipid composition, secretion and bile acid pool size have been measured in obese subjects, before and after weight reduction. Weight reduction was found consistently to lead to decreased cholesterol content of bile and reduced cholesterol secretion rate, while bile acid and lecithin secretion were unchanged. These results suggest that weight control may reduce the

incidence of gallstones and in the obese may prevent recurrences of gallstones if dissolution can be achieved during or after weight reduction. (Drs. Bennett and Bennion)

Since oestrogens have been implicated in the etiology of human gallstones biliary lipid composition was measured during different phases of the menstrual cycle. No significant differences in composition were found during normal menstrual cycles, but further information is needed to determine whether or not exogenous oestrogens may cause bile composition changes. (Drs. Bennett, Bennion and Ginsberg)

The Section has investigated methods which may lead to the dissolution of gallstones and the effects of cholecystectomy on bile composition. Lithogenicity of hepatic bile had been reported reduced following cholecystectomy. This conclusion could not be confirmed by investigations carried out in this Section and these results are being published. (Drs. Grundy, Adler and Metzger)

Oral administration of chenodeoxycholic acid in doses of less than 1 gm/day was shown to significantly change biliary lipid composition, to significantly reduce the lithogenicity of bile and, rather unexpectedly, to reduce the rate of cholesterol secretion. These findings indicate that therapeutic effects, probably sufficient for gallstone dissolution, may be achieved with low doses of chenodeoxycholic acid and that chenodeoxycholic acid may not be simply a "replacement therapy", but may also perhaps inhibit cholesterol synthesis. (Drs. Bennett, Bennion, Duane and Adler)

Animal studies have indicated that phenobarbital may increase bile salt synthesis and flow rates, and reduce the cholesterol saturation of bile. The effects of phenobarbital on bile composition, pool size and secretion rates in normal volunteers are being studied to determine whether the physiologic effects in animals are reproduced in man. If so phenobarbital may prove to be a desirable therapy for gallstones in man. (Drs. Bennett and Ginsberg)

The mechanism of cholesterol synthesis and its relationship to gallstone formation in animals has been investigated. HMG CoA reductase, the rate limiting enzyme in cholesterol synthesis was determined in genetically obese rats and nonobese sibling controls. While no difference in concentrations of HMG CoA reductase/mg microsomal protein were shown, the total HMG CoA activity in the obese-hypercholesterolemic animals was much greater. The assay has been performed on hamsters fed a lithogenic diet and controls. Gallstone formation has occurred, but results are too few to draw any overall conclusions at this time. (Drs. Flock, Duane and Bennett)

Alcohol Metabolism

Previous reports suggested that reduced rates of alcohol metabolism were characteristic of American Indians. Alcohol tolerance tests have been performed in Indian and control subjects, but no difference in clearance rates was found. Alcohol dehydrogenase activity in liver has also been compared,

but no significant difference was found, although a previously unknown isoenzyme has been identified among the Indian subjects. (Drs. Bennett, Bennion and Li)

During the past year significant staff reduction of the Section has taken place, so that at the present time, only 7 full-time and 2 part-time positions remain of the previously authorized 18 positions. This development will certainly further constrict the activities of the Section in the coming year.

Serial No. NIAMDD-DDB-1 (c)
1. Digestive Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Mechanisms of Secretion in Human Eccrine Sweat Glands

Previous Serial Number: NIAMDD-MDB-7 (c)

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators: Dr. James W. Benson, Jr. and Mrs. Angela Chu

Cooperating Units: Surgical branches of NHLI and NCI and Section on Pathology, NHLI

Man Years:

Total	: 1.75
Professional	: .75
Other	: 1.00

Project Description:

Human sweat glands are extraordinary in their ability to transfer large quantities of isotonic fluid from blood to lumen. They are also unusual in having a very high rate of anerobic glycolysis, and appear unable to base their metabolism on oxidative degradation of fats. Detailed study of the relationship of metabolism to secretory function in this tissue might lead ultimately to additional understanding of the process by which isotonic fluid is transported by epithelia.

Methods and Major Findings: Studies during this fiscal year have depended primarily on incubation of surviving human sweat glands in vitro. The tissue is obtained by microdissection, using vital staining, of individual sweat glands from minute pieces of human skin obtained in the course of surgery. A considerable problem that still remains is the difficulty of freeing sweat glands entirely from adherent fat cells. A number of experiments with collagenase were disappointing, since treatment adequate to remove all fat appeared to impair tissue metabolism. Mechanical dissection on a cell-by-cell basis requires so much time that it is impossible to obtain enough tissue for a study. Therefore, reluctantly, we have had to carry out incubations with a known contamination of sweat gland tissue with adipocytes.

Tissue surviving in vitro will convert ^{14}C-glucose to ^{14}C-lactic acid at a measurable rate even in the presence of oxygen. Ion exchange column chromatography has been adapted to separate the labeled product from residual labeled glucose, so that radiochemical measurement of lactate production in vitro can be used as an index of sweat gland metabolism. Studies currently under way are designed to confirm older results, based on chemical measurement of lactate production, which indicated that glycolysis could be stimulated in vitro by mecholyl or epinephrine. We have also begun experiments to try to detect an effect of insulin on uptake of glucose or its metabolism by sweat glands.

Although no new experiments have been undertaken, time has been spent during the current year in the preparation of two manuscripts based on last year's work. One, describing the experiments reported last year on the effect of starvation on the function and histochemistry of human sweat glands, has been accepted for publication. The other, summarizing the work on transfer of ^{14}C-urea from blood to sweat, is in preparation.

Proposed Course of Project: It is anticipated that this project will be discontinued as soon as the results of experiments now in progress can be published.

Significance to Bio-Medical Research and Program of the Institute: The processes whereby exocrine glands elaborate and transport their secretory product are of very general interest and are still imperfectly understood. It is hoped that our studies will throw some additional light on the secretory mechanisms within the sweat gland, which may in turn be useful in interpreting the process of secretion in general. More particularly, since cystic fibrosis, a major area of program concern to NIAMDD, is clearly manifest as an abnormality of sweat secretion, we always consider the possibility that further progress in understanding the secretion of normal sweat glands may throw light on the basic inherited defect of cystic fibrosis.

Honors and Awards: None.

Publications: None.

Serial No. NIAMDD-DDB-2 (c)
1. Digestive Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Prospective Clinical Trial of Intestinal Lavage as a Preparation for Diagnostic X-ray Studies

Previous Serial Number: None

Principal Investigators: Dr. Robert S. Gordon, Jr., NIAMDD
Dr. Jean Herdt, Diagnostic X-ray, CC

Other Investigators: Dr. James W. Benson, Jr., NIAMDD
Dr. Arnold G. Levy, NIAMDD
Dr. John L. Doppman, CC
Dr. Michael Vermess, CC

Cooperating Units: Diagnostic Radiology Department, Clinical Center

Man Years:

Total	:	.75
Professional	:	.50
Other	:	.25

Project Description:

Barium enema is a familiar radiologic diagnostic procedure, one which is of great importance in the diagnosis and study of a number of diseases. Perhaps the most significant of these is colonic cancer which still ranks among the leading causes of death in the United States, but which should be almost completely curable if it could routinely be detected at an early stage. Before a successful barium enema can be carried out, it is necessary that the patient's colon be cleansed of all feces and contents; if this process is not thorough the radiologic examination is unsatisfactory. Adequate preparation, assuring an X-ray of high quality, is frequently very difficult for the patient, since it requires drastic catharsis and multiple enemas. The purpose of this project is to evaluate the acceptability both for the patient and for the radiologist of an alternate method of preparation.

Methods and Findings: If a human subject voluntarily ingests an isotonic balanced salt solution at a high rate (2 or more liters per hour), the maximum capacity of the small intestine for the absorption of such fluid will be exceeded. The excess will then pass distally, where it lavages the colon and is passed normally per ano. If saline is consumed over a period of two to three hours, the volume excreted will be sufficient, in most cases, to remove all fecal material from the lower digestive system.

Patients throughout the Clinical Center, who are scheduled for a barium enema as part of their diagnostic or follow-up study, are being considered for inclusion in a randomized clinical trial. Those who meet certain criteria and agree to cooperate are assigned at random to be prepared either by the accepted standard procedure using castor oil and enemas, or by the experimental procedure outlined above. Needless to say, this clinical trial cannot be kept double blind, but the radiologist who performs and interprets the barium enema is not told which method of preparation was used. After each procedure, both the radiologist and the patient evaluate the efficacy and tolerability of the preparation procedure by answering a standard questionnaire.

The number of patients studied is not sufficient for firm conclusions, but a trend is already evident in the results. A few patients find it impossible to consume an adequate volume of saline, so that for them the experimental method is not applicable. The remainder, who are successful, for the most part rate the saline drinking method as being more comfortable and less troublesome than the alternate. From the point of view of the radiologists, there seems to be little difference between the two. Additional measurements of blood chemistries indicate that whereas castor oil produces a mild degree of dehydration, and saline a transient overhydration, neither method alters any of these parameters to a significant degree.

Proposed Course of Project: The project will be continued probably for another six months, until the number of patients is large enough for firm conclusions to be drawn.

Significance to Bio-Medical Research and Program of the Institute: It is apparent that any improvement in the efficacy and/or acceptability of the barium enema would be an advantage in the diagnosis and study of essentially any disease affecting the colon in man.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-3 (c)
1. Digestive Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Therapeutic Trial of Phenobarbital as a Cholelitholytic Agent

Previous Serial Number: None

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators: Dr. James W. Benson, Jr.

Cooperating Units: Diagnostic X-ray Department, Clinical Center

Man Years:

Total	:	.75
Professional	:	.50
Other	:	.25

Project Description:

It is now well accepted that cholesterol gallstones can be dissolved *in situ* in selected patients. At least in the short term, this has made cholecystectomy unnecessary for the treatment of gallstone disease. However, the one agent which has so far been shown to be effective, chenodeoxycholic acid (CDCA), has certain drawbacks. It is still in short supply and relatively expensive. There are certain minor annoying side effects, and one side effect which may be of greater concern, the tendency of the agent to induce abnormal liver chemistries in patients taking the doses that have so far been found to be effective. For these reasons, there is considerable interest in a search for other medications which might have the same desirable effect without the drawbacks of CDCA.

Recent metabolic studies in animals, which are now being extended to human volunteers in NIAMDD's Clinical Research Section in Phoenix, indicate that chronic administration of phenobarbital, presumably by its inducing effect on hepatic microsomal enzyme systems, will increase the rate of endogenous bile acid secretion. These studies suggest the possibility that chronic phenobarbital administration, in this indirect way,

might have an effect similar to that of CDCA feeding. The present project constitutes a controlled clinical trial of that possibility.

Methods: Patients are being recruited who fulfill the following criteria: 1) Age 40 to 70, 2) radiolucent gallstones in a functioning gallbladder, and 3) absence of symptoms severe enough to indicate early surgery. Those who are willing to participate in the therapeutic trial are being randomized, and given either phenobarbital or a placebo. Every patient receives 50 mgm of ascorbic acid per day to rule out the possibility of subclinical vitamin C deficiency, since such deficiency may impair the activity of hepatic cholesterol-7-alpha-hydroxylase. All patients will be seen at intervals in the outpatient department, and cholecystograms will be repeated at 6, 12, and 24 months. If there is no change in the size or number of gallstones over a two-year period, the patient will be considered a therapeutic failure. The basic criterion for success will consist of radiologic demonstration of a significant decrease in the size and number of gallstones.

Results: It is too early for any results to be available.

Proposed Course of Project: We anticipate that the whole study will take approximately three years and will continue as outlined above.

Significance to Bio-Medical Research and Program of the Institute: Cholesterol gallstone disease is a major cause of morbidity in the United States, and the economic cost is enormous. A simple and acceptable medical method for dissolving gallstones without surgery might have a major impact in reducing the cost of this condition, both in dollars and in suffering, to the American public.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-4 (c)
1. Digestive Diseases Branch
2. Section on Physiology and Clinical Nutrition
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Total Energy Metabolism and Temperature Regulation: Studies in Health and Disease

Previous Serial Number: NIAMDD-MDB-6 (c)

Principal Investigator: Dr. Robert S. Gordon, Jr.

Other Investigators: Dr. Ronald H. Thompson and Mr. Del Thrasher

Cooperating Units: Laboratory of Clinical Investigation, NIAID
Reproduction Research Branch, NICHHD
Laboratory of Clinical Investigation, NINDS

Man Years:

Total	:	2.25
Professional	:	1.25
Other	:	1.00

Project Description:

Objectives: To elucidate the mechanisms of normal physiologic response, particularly temperature regulation, to various stimuli including climatic stress, and to investigate the characteristics of temperature regulation, and aberrations of this function in a variety of disease conditions.

Methods Employed: Total body heat loss and temperature regulation by thermoelectric and radiometric techniques under controlled environmental conditions, and precise measurement of evaporative and metabolic weight loss.

Accomplishments: The environmental chamber itself has been in use for studies of sweat secretion (q.v.). In addition, the chamber has been used extensively for studying the thermoregulatory response of patients on other services to extremes of heat and cold. Two patients from NIAMDD were studied. In addition, 10 patients with fevers of unknown origin, being studied on the NIAID service, have been handled in the chamber. NINDS referred one case, a patient with loss of sudomotor control, caused by sympathetic nerve degeneration, for studies of thermoregulation. An additional 83 studies have been done in collaboration with investigators in NICHHD who are

carrying out research on the nature and causes of anorexia nervosa. It has been found that while most patients with hypothalamus-pituitary gland-target organ defects are able to regulate their body temperatures when exposed to either heat or cold, patients with anorexia nervosa show a marked defect in this control. This appears to persist even in cases where a remission occurs, and the patient regains normal weight.

Significance to Bio-medical Research and Program of the Institute: Same as for the past four years.

Proposed Course of Project: Dr. Gordon having left NIAMDD's Section on Physiology and Clinical Nutrition to assume directorship of the Clinical Center, the future of this project is in doubt at the time of this writing.

Honors and Awards: None

Publications:

1. Mecklenberg, R.S., Loriaux, D.L., Thompson, R.H., Anderson, A.E., and Lipsett, M.B.: Hypothalamic dysfunction in patients with anorexia nervosa. Medicine 53:147-59, 1974.

Serial No. NIAMDD-DDB-5 (c)
1. Digestive Diseases
2. Diseases of the Liver
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Removal of organic anions from blood by hemoperfusion.

Previous Serial Number: None

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: Bruce F. Scharschmidt, M.D., James F. Martin, M.D.

Cooperating Units: Arthritis and Rheumatism Branch, NIAMDD (Dr. Paul Plotz)
Pregnancy Research Branch, NICHD (Dr. Ronald Chez)

Man Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives:

To develop methods for removing from the blood those metabolic products which are ordinarily extracted by the liver. The ultimate objective of this project is the development of an artificial support system for use in the management of hepatic failure.

Methods Employed:

To date we have concentrated on the removal of bilirubin from blood. Bilirubin was selected as a test substance because of its extremely high binding to serum proteins, which makes it a particularly difficult substance to remove. We have used the approach of affinity chromatography, using human serum albumin conjugated in high concentration to agarose beads as a prototype of a simple "artificial liver" for the removal of organic anions from the circulation.

Major Findings:

1. Affinity chromatography using albumin agarose beads is a highly efficient means of removing bilirubin, certain bile acids, BSP, indocyanine green and a number of other physiologic metabolites from the circulation.

2. The albumin agarose beads appear to be highly compatible with whole blood and prolonged hemoperfusion both *in vitro* and *in vivo* in animals has minimal effect on formed elements of the blood.

3. In vivo studies in jaundiced rats have demonstrated that one hour of hemoperfusion over an appropriate size column was sufficient to remove virtually the entire circulating plasma bilirubin pool. This is true in both jaundiced Gunn rats who have hereditary unconjugated hyperbilirubinemia and in Sprague-Dawley rats with ligated bile ducts who have conjugated hyperbilirubinemia. Extrapolation from the kinetics of bilirubin removal in rats suggests that three 6-hour hemoperfusions per week would remove the entire bilirubin load produced during this time from a patient with total hepatic failure.

Significance to Biomedical Research:

The ability to support the patient in hepatic failure by means of an artificial system for the removal of waste products would have obvious clinical implications. Even the simple system developed at this point, which removes predominantly bilirubin, has a number of applications. Jaundice in the first week following birth is extremely common and may result from a variety of causes. Although usually benign, severe jaundice during the early neonatal period can result in significant brain damage and the resultant clinical syndrome of kernicterus. Current treatment of neonatal jaundice is limited to phototherapy in mild cases, and exchange transfusion in severe cases. Although phototherapy appears to be safe, long term follow-up studies of its possible side effects are not yet available. In addition, for reasons not understood, it fails to work in a number of cases. Exchange transfusion, even in the best of hands, carries with it an approximately 1% mortality rate and significant morbidity, including the possibility of transmitting blood-borne diseases such as hepatitis. Hence, hemoperfusion of blood of newborns over albumin agarose beads may offer a safe and effective alternative means of treating neonatal hyperbilirubinemia. The availability of umbilical vessels as both sites of entry and exit to the circulation would make the mechanics of hemoperfusion in the newborn period extremely simple. In addition, the potential use of hemoperfusion over albumin agarose columns for the management of diseases characterized predominantly by increases in bilirubin and/or bile salt concentrations, such as Crigler-Najjar syndrome or congenital biliary atresia, is also under study.

Proposed Course:

We plan to evaluate the practical utility of albumin agarose columns for the management of hyperbilirubinemia in the following situations:

A protocol has been designed which would permit us to perform hemoperfusion in premature newborn Rhesus monkeys delivered by caesarian section. These animals develop neonatal hyperbilirubinemia similar to that seen in human newborns. This study will permit assessment of the safety and efficacy of hemoperfusion in the newborn period. In parallel with this study we will perform hemoperfusion in newborn lambs. Although sheep do not get neonatal hyperbilirubinemia, newborn lambs are of approximately the same birth weight as human newborns. In addition, because their size is considerably greater than that of newborn monkeys, permitting greater blood sampling, it will be possible to assess in detail various other physiologic effects of hemoperfusion, such as possible depletion of hormones and fatty acids. It is hoped that the successful completion of a detailed evaluation of hemoperfusion in newborn lambs and monkeys will permit a decision as to whether or not this

procedure appears to be safe and suitable for trial in human neonatal hyperbilirubinemia. Simultaneously with these studies of neonatal hyperbilirubinemia, which are being performed in collaboration with Dr. Paul Plotz of the Arthritis and Rheumatism Branch of NIAMDD and Dr. Ronald Chez of the National Institute of Child Health and Human Development, we will be continuing to work on improvement of the albumin agarose columns both in terms of their flow properties and their bilirubin capacity. As a new aspect of the project beginning in July of 1974, we intend to begin studying the properties of a variety of synthetic resins, both charged and uncharged, for the removal of other metabolites which accumulate in the circulation during the course of more generalized hepatic failure. In addition, intense studies are going on to develop a reproducible model of acute, reversible hepatocellular failure in order to serve as a trial for the evaluation of hepatic support systems.

Honors: None.

Publications:

Plotz, P.H., Berk, P.D., Scharschmidt, B.F., Gordon, J.K. and Vergalla, J.: Removing substances from blood by affinity chromatography. I. Removing bilirubin and other albumin bound substances from plasma and blood with albumin-conjugated agarose beads. J. Clin. Invest. 53: 778-785, 1974.

Scharschmidt, B.F., Plotz, P.H., Berk, P.D., Waggoner, J.G. and Vergalla, J.: Removing substances from blood by affinity chromatography. II. Removing bilirubin from the blood of jaundiced rats by hemoperfusion over algumin-conjugated agarose beads. J. Clin. Invest. 53: 786-795, 1974.

Serial No. NIAMDD-DDB-6 (c)
1. Digestive Diseases
2. Diseases of the Liver
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of the Transport of Organic Anions by the Liver

Previous Serial Number: None

Principal Investigator: Paul D. Berk, M.D.

Other Investigators: James F. Martin, M.D., Bruce F. Scharschmidt, M.D.

Cooperating Units: None

Man Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives:

1) To study the transport of organic anions from blood through the liver into bile, in order to characterize the biophysical nature of each of the transport steps involved in the overall hepatic excretory system.

2) To develop a capability for quantitating the function of each of these steps in patients with hepatic dysfunction by analysis of kinetic studies of various test substances such as sulfobromophthalein, indocyanine green and radiolabeled bilirubin.

Methods Employed:

Unconjugated bilirubin-^{14}C or -^{3}H is produced biosynthetically in dogs with external biliary fistulae using δ-amino levulinic acid-^{14}C or -^{3}H as a precursor. After appropriate crystallization and filtration to render the product sterile and pyrogen-free, radiolabeled bilirubin is injected intravenously into patients or animals in order to determine plasma radiobilirubin disappearance curves. Analysis of these curves permits direct calculation of both hepatic bilirubin clearance (C_{BR}: ml/min/kg) and plasma bilirubin turnover (BRT: μmols/kg/day). Further analysis of the disappearance curves in terms of a previously proposed compartmental model of bilirubin metabolism permits direct calculation of such parameters of physiologic interest as hepatic bilirubin uptake rate, the intrahepatic bilirubin concentration, the liver to plasma bilirubin concentration gradient and the hepatic bilirubin storage capacity. Similar kinetic analyses of plasma disappearance curves of sulfobromophthalein and indocyanine green are also performed.

Major Findings:

Considerable effort has been expended in characterizing the abnormalities of bilirubin metabolism observed in patients with a number of hereditary diseases, such as Gilbert's syndrome and Crigler-Najjar syndrome. Understanding of the defects present in these "experiments of nature" sheds considerable light on normal human hepatic function. Gilbert's syndrome is a common hereditary abnormality characterized by mild unconjugated hyperbilirubinemia which we have shown results from a reduction in hepatic bilirubin clearance. Studies by others have clearly demonstrated that levels of the bilirubin conjugating enzyme, bilirubin UDP-glucuronyl transferase, are reduced in the livers of these patients, but it remains uncertain whether or not this reduction in in vitro assays of glucuronyl transferase explains the in vivo defect in bilirubin clearance. We have investigated this phenomenon in several ways. First, studies of bilirubin kinetics in patients with Gilbert's syndrome, both before and during treatment with enzyme inducing drugs, have demonstrated that both phenobartibal and glutethimide produce approximately a three-fold increase in hepatic bilirubin clearance in patients with Gilbert's syndrome, and, in fact, result in clearances within the normal range. A smaller increase in bilirubin clearance results from similar treatment of normal volunteers. While measurements of UDP-glucuronyl transferase, performed either in our laboratory or elsewhere, in patients with Crigler-Najjar syndrome, Gilbert's syndrome, untreated normal controls and phenobarbital treated normal controls correlate highly with measurements of bilirubin clearance made in corresponding groups, the marked increase in bilirubin clearance observed in patients with Gilbert's syndrome during phenobarbital or glutethimide treatment is not accompanied by a corresponding increase in measurements of glucuronyl transferase activity in liver tissue from similar patients. Thus, although bilirubin clearance becomes normal in patients with Gilbert's syndrome during phenobarbital or glutethimide therapy, this normalization of in vivo bilirubin metabolism as measured in our laboratory is not accompanied by a normalization of bilirubin conjugating enzyme levels, as measured in several other institutions. This suggests clearly that the reduction in bilirubin UDP-glucuronyl transferase cannot totally explain the abnormalities in bilirubin kinetics and in vivo bilirubin metabolism observed in Gilbert's syndrome.

Although several reviews of Gilbert's syndrome indicate that all other tests of liver function in such patients are normal, we have studied individuals with unconjugated hyperbilirubinemia and normal liver biopsies in whom in vivo studies of radiobilirubin kinetics are identical to those of typical Gilbert's syndrome, but in whom there are abnormalities of BSP metabolism. Although the majority of patients with Gilbert's syndrome do have normal BSP metabolism we have characterized two sub-groups, one of which has an apparent defect in the hepatic uptake of BSP as determined by compartmental analysis of BSP disappearance curves. The second sub-group has a defect at a later stage in BSP transport, the nature of which has not been clarified further. Indocyanine green is a water soluble tricarbocyanine dye which is being increasingly used as a test of liver function. In the standard test, 0.5 mg/kg of indocyanine green is injected intravenously and serial samples are obtained for approximately 20 minutes in order to determine the fractional disappearance rate, or half-life, of the injected dye. Studies in our laboratory have indicated that indocyanine green clearance is normal in all patients with

Gilbert's syndrome, irrespective of the presence or absence of abnormalities in BSP metabolism, when the standard 0.5 mg/kg dose of indocyanine green is employed. We have now performed studies of the disappearance rates of multiple doses of indocyanine green ranging from 0.5 to 5.0 mg/kg in a large population of normal volunteers and patients with Gilbert's syndrome. In normal individuals and in those patients with Gilbert's syndrome who have normal BSP kinetics, increasing the dose of indocyanine green from 0.5 to 5.0 mg/kg results in only a very small reduction in the fractional disappearance rate of dye from plasma. In contrast, in those patients who have an abnormally slow rate of BSP disappearance from plasma the increase in indocyanine green dose from 0.5 to 5.0 mg/kg is accompanied by a dramatic fall in fractional ICG disappearance rate. A plot of ICG removal rate vs. dose is clearly indicative of saturation kinetics. Michaelis-Menten analysis of these indocyanine green studies demonstrates that there is a sub-class of patients with Gilbert's syndrome in whom the V_{max} for indocyanine green uptake into the liver is reduced to 1.5 mg/min/kg of body weight, compared to a normal mean value of 4.7. The demonstrated abnormalities in indocyanine green and sulfobromophthalein metabolism, both of which are metabolized by pathways which do not involve glucuronide conjugation, clearly demonstrates that at least in some patients with Gilbert's syndrome there are abnormalities of organic anion transport not attributable to defects in bilirubin UDP-glucuronyl transferase.

In another series of studies of bilirubin metabolism, bilirubin turnover and bilirubin clearance were both calculated before and after splenectomy in patients with hereditary spherocytosis. Following splenectomy bilirubin turnover uniformly fell in all patients with hereditary spherocytosis, reaching an average value of approximately 20% of the pre-splenectomy result. In all cases this was associated with a marked prolongation of mean red cell survival and a fall in plasma bilirubin concentration. Comparison of pre-operative and post-operative values for bilirubin clearance demonstrated that in those individuals whose pre-operative bilirubin clearance was normal the marked fall in bilirubin turnover associated with splenectomy produced no change in the rate of hepatic bilirubin clearance. In contrast, in those patients who pre-operatively had a hepatic defect in bilirubin metabolism of the Gilbert's type the marked reduction in bilirubin turnover associated with splenectomy was associated uniformly with an increase in bilirubin clearance. This suggests that the overall pathway for bilirubin removal in patients with Gilbert's syndrome is operating at a level close to saturation. Which particular step in the overall transport process is responsible for this saturation will be determined in future studies.

The relationship between bilirubin turnover, representing the load of bilirubin presented to the liver daily for removal, and hepatic bilirubin clearance, was also examined in a patient with Crigler-Najjar syndrome. In this condition marked hyperbilirubinemia due to a complete absence of bilirubin glucuronyl transferase results in a constant risk of brain damage. In one genetic variant of this syndrome hyperbilirubinemia can be alleviated by treatment with enzyme inducing drugs, such as phenobarbital. In another variant, however, there is no response to any pharmacologic modality. Accordingly, in such a patient we explored the possibility that a reduction in bilirubin turnover would result in a proportional fall in plasma bilirubin

concentration and reduce the risk of brain damage. A 22-compartment model of erythrokinetics was developed in order to simulate the effects of chronic phlebotomy on erythrokinetics and bilirubin production. Control studies of bilirubin clearance, bilirubin turnover, red cell life span, circulating red cell mass and bone marrow hemoglobin pool were analyzed in terms of this computer model in order to predict the effects of a 500 ml per week phlebotomy. The computer model predicted that phlebotomy would be associated with a marked fall in bilirubin turnover due to the skewing of the red cell age distribution toward young cells. It was expected that this fall in bilirubin turnover would be associated with a corresponding fall in bilirubin concentration in plasma. Accordingly, a patient with Crigler-Najjar syndrome was phlebotomized for a four month period. In contrast to computer predictions, the plasma bilirubin concentration did not fall. A repeat of the various studies listed above indicated that the computer model's predictions of the effect of phlebotomy on red cell age distribution, red cell survival, circulating and marrow hemoglobin pools and bilirubin turnover, had all been highly accurate. It was found, however, that the fall in bilirubin turnover in Crigler-Najjar syndrome was associated with a corresponding fall in bilirubin clearance, cancelling out any possible therapeutic benefit of the decreased rate of bilirubin synthesis. Although these studies produced no therapeutic benefit for the patient they indicated that the various alternate pathways of bilirubin excretion in patients with Crigler-Najjar syndrome are uniquely dependent on the rate of bilirubin turnover and appear to have a threshhold effect.

Significance to Biomedical Research:

Studies of organic anion kinetics in patients with Gilbert's syndrome, Crigler-Najjar syndrome and normal volunteers have led to a marked improvement in our understanding of the excretory function of the liver. In particular these studies have demonstrated that it is possible to quantitate hepatic function in terms of clearances in the manner analogous to the use of creatinine clearance measurements for quantitating renal function. The ability to accurately measure both bilirubin turnover and bilirubin clearance, the two parameters which determine the plasma bilirubin concentration, has made possible a precise functional classification of all cases of unconjugated hyperbilirubinemia. Studies of the sort described above are also leading both to the development of new clinical methods for assessing the function of individual steps in organic anion transport processes and to an improved understanding of the nature of the defects in a variety of disease states.

Proposed Course:

We plan to continue our evaluation of organic anion transport in patients with Gilbert's syndrome. In particular new approaches to studies of bilirubin kinetics involving the intravenous injection of various loading doses of bilirubin, in addition to tracer doses, will be employed in an attempt to see whether or not those patients with demonstrated abnormalities in the hepatic uptake of indocyanine green and bromosulfophthalein can also be shown to have a corresponding alteration in the hepatic uptake of bilirubin. In addition, the methods currently used for the studies of unconjugated kinetics will be extended to permit studies of the fate of conjugated radiolabeled bilirubin. This will permit the application of these isotope techniques to a variety of more common disease states, including cirrhosis and

viral hepatitis.

Honors: None

Publications:

Berk, P.D., Berlin, N.I. and Howe, R.B.: Disorders of bilirubin metabolism. In Bondy, P.K. and Rosenbert. L. (Eds.): Duncan's Diseases of Metabolism. Philadelphia, Pa., W. B. Saunders and Co., 1974, pp. 825-880.

Berk, P.D., Rodkey, F.L., Blaschke, T.F., Collison, H.A. and Waggoner, J.G.: Comparison of plasma bilirubin turnover and carbon monoxide production in man. J. Lab. Clin. Med. 83: 29-37, 1974.

Berk, P.D., Berlin, N.I., Scharschmidt, B.F. and Blaschke, T.F.: Hepatic bilirubin clearance (C_{BR}) in the differential diagnosis of unconjugated hyperbilirubinemia: I. Estimation of C_{BR} from studies with radiochromium. Epatologia (in press).

Berlin, N.I. and Berk, P.D.: The biological life span of the red blood cell. In Surgenor, D.Mac N. (Ed.): The Red Blood Cell. New York, N. Y., Academic Press, 1974 (in press).

Berk, P.D., Blaschke, T.F. and Rodkey, F.L.: Effects of phenobarbital and glutethimide on heme turnover, plasma bilirubin turnover and hepatic bilirubin clearance in man. In Paumgartner, G. and Preisig, R. (Eds.): The Liver: Quantitative Aspects of Structure and Function. Basel, Switzerland, S. Karger, 1973, pp. 184-193.

Bloomer, J.R., Berk, P.D., Howe, R.B.: Hepatic clearance of unconjugated bilirubin in cholestatic liver diseases. Amer. J. Dig. Dis. 19: 9-14, 1974.

Blaschke, T.F., Berk, P.D., Scharschmidt, B.F., Guyther, J.R., Vergalla, J.M. and Waggoner, J.G.: Crigler-Najjar syndrome: An unusual course with development of neurologic damage at age 18. Pediatric Res. (in press).

Gisselbrecht, C. and Berk, P.D.: Failure of phenobarbital to increase bilirubin production in the rat. Biochem. Pharmacol. (in press).

Blaschke, T.F., Berk, P.D., Rodkey, F.L., Scharschmidt, B.F., Collison, H.A. and Waggoner, J.G.: Drugs and the liver. I. Effects of glutethimide and phenobarbital on hepatic bilirubin clearance, plasma bilirubin turnover and carbon monoxide production in man. Biochem. Pharmacol. (in press).

Bloomer, J.R., Berk, P.D., Vergalla, J. and Berlin, N.I.: Influence of albumin on the hepatic uptake of unconjugated bilirubin. Clin. Sci. 45: 505-516, 1973.

Bloomer, J.R., Berk, P.D., Vergalla, J. and Berlin, N.I.: Influence of albumin on the extravascular distribution of unconjugated bilirubin. Clin. Sci. 45:517-526, 1973.

Schwartz, S., Stephenson, B.D., Sarkar, D., Bossenmaier, I., Cardinal, R.A., Weimer, M., Swaim, W. R. and Berk, P.D.: Isotopic studies of porphyrin and heme metabolism: Bonanzas and pitfalls. Ann. N.Y. Acad. Sci. (in press: fall 1974).

Serial No. NIAMDD-DDB-7(c)
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Immunophysiology of the Human Small Intestine

Previous Serial Number: NIAMDD-DHDB-1(c)

Principal Investigator: Dr. Jerry Gardner

Other Investigators: Drs. Hayden L. Klaeveman and Arnold G. Levy, NIAMDD; Mrs. Clementine Sessoms, NIAMDD: Dr. Warren Strober, NCI; and Dr. A. J. Tousimis (Biodynamics Research Corp.).

Cooperating Units: National Cancer Institute
Biodynamics Research Corp. (Contract NO1-AM-3-2202)

Man Years:

Total:	1.75
Professional:	1.0
Others:	.75

Project Description:

We have continued our studies of the characteristic features of the immune system in the human small intestine and effects of disease on these various processes. By measuring the effect of dietary gluten on small intestinal immunoglobulin synthesis we have been able to readily identify patients with gluten-sensitive enteropathy. By studying the immunologic function of the small intestinal mucosa from patients with ulcerative ileojejunitis we found that this disease appears to constitute an "autoimmune" variant of sprue.

Honors and Awards: None

Publications:

Warshaw, A. L., Waldmann, T. A., and Laster, L.: Protein-losing enteropathy and malabsorption in regional enteritis. Cure by limited ileal resection. Annals Surg. 178: 578-580, 1973.

Warshaw, A. L., and Laster, L.: Protein synthesis by human intestinal mucosa: Variations with diseases of the gut. J. Surg. Res. 14: 285-293, 1973.

Gebhard, R. L., Katz, S. I., Marks, J., Shuster, S., Trapani, R. J., Rogentine, G. N., and Strober, W.: HL-A Antigen type and small-intestinal diseases in dermatitis herpetiformis. Lancet 2: 760-762, 1973.

Agus, S. G., Falchuk, Z. M., Sessoms, C. S., Wyatt, R. G., and Dolin, R.: Increased jejunal IgA synthesis in vitro during acute infectious nonbacterial gastroenteritis. Am. J. Dig. Dis. 19: 127-131, 1974.

Agus, S. G., Dolin, R., Wyatt, R. G., Tousimis, A. J., and Northrup, R. S.: Acute infectious nonbacterial gastroenteritis: Intestinal histopathology. Ann. Int. Med. 79: 18-25, 1973.

Falchuk, Z. M., Gebhard, R. L., Sessoms, C., and Strober, W.: An in vitro model of gluten-sensitive enteropathy. Effect of gliadin on intestinal epithelial cells of patients with gluten-sensitive enteropathy in organ culture. J. Clin. Invest. 53: 487-500, 1974.

Falchuk, Z. M., and Leventhal, B. G.: Budd-Chiari syndrome in a patient with paroxysmal nocturnal hemoglobinuria. Am. J. Dig. Dis. 18: 900-904, 1973.

Gebhard, R. L., Falchuk, Z. M., Katz, S. I., Sessoms, C., Rogentine, G. N., and Strober, W.: Dermatitis herpetiformis: Immunologic concomitants of small intestinal disease and relationship to histocompatibility antigen HL-A8. J. Clin. Invest. In press.

Serial No. NIAMDD-DDB-8(c)
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Biochemical Aspects of Disease

Previous Serial Number: NIAMDD-DHDB-3(c)

Principal Investigator: Dr. Leonard Laster

Other Investigators: Dr. Jeffery R. Pool and Mr. William A. Edwards, NIAMDD:
Dr. S. Harvey Mudd, NIMH

Cooperating Units: National Institute of Mental Health

Project Description:

This project was discontinued July 1, 1973.

Serial No. NIAMDD-DDB-9(c)
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of Membrane Function

Previous Serial Number: NIAMDD-DHDB-4(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Hayden L. Klaeveman and Arnold G. Levy, NIAMDD; Mrs. Clementine Sessoms, NIAMDD; Dr. John R. Gill, Jr., NHLI; and Dr. Vincent P. Butler, Jr., Columbia University College of Physicians and Surgeons, N.Y.

Cooperating Units: National Heart and Lung Institute
Columbia U. College of Physicians and Surgeons, N.Y.

Man Years:

Total:	1.25
Professional:	.75
Others:	.5

Project Description:

The types of studies which are included under this project are: 1) Characterizing functionally the mechanism by which various substrates cross the plasma membrane of different mammalian cells; 2) identifying the metabolic and humoral factors which influence the transport of various substrates across the plasma membrane; 3) developing techniques which will distinguish between binding of a substrate to the membrane and translocation of the substrate across the membrane; 4) characterizing the mechanism by which the membrane transport of various substrates is altered in certain diseases; and 5) relating these alterations of membrane transport to the pathogenesis and clinical manifestations of the disease.

We have solubilized and characterized functionally the plasma membrane receptor for cardioactive glycosides. Future plans are to investigate the chemical characteristics of this receptor.

Honors and Awards: None

Publications:

Gardner, J. D., Kiino, D. R., Swartz, T. J., and Butler, V. P., Jr.: Effects of digoxin-specific antibodies on accumulation and binding of digoxin by human erythrocytes. J. Clin. Invest. 52: 1820-1833, 1973.

Gardner, J. D., and Kiino, D. R. Ouabain binding and cation transport in human erythrocytes. J. Clin. Invest. 52: 1845-1851, 1973.

Butler, V. P., Jr., Watson, J. F., Schmidt, D. H., Gardner, J. D., Mandel, W., and Skelton, C. L.: Reversal of the pharmacological and toxic effects of cardiac glycosides by specific antibodies. Pharmacol. Rev. 25: 239-248, 1973.

Gardner, J. D., and Frantz, C.: Effects of cations on ouabain binding by intact human erythrocytes. J. Memb. Biol. 16: 43-64, 1974.

Butler, V. P., Jr., Schmidt, D. H., Watson, J. F., and Gardner, J. D.: Production and properties of digoxin-specific antibodies. Ann. N. Y. Acad. Sci. In press.

Serial No. NIAMDD-DDB-10(c)
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Gastrointestinal Hormones

Previous Serial Number: NIAMDD-DHDB-5(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Dr. Hayden L. Klaeveman, Mr. Trent D. Adams and Mr. Thomas P. Conlon, NIAMDD

Cooperating Units: None

Man Years:

Total:	1.75
Professional:	.5
Others:	1.25

Project Description:

In vitro systems are being used to study the mechanism of action of gastrin, secretin, cholecystokinin and vasoactive polypeptide hormone with their specific membrane receptors.

Honors and Awards:

A paper entitled "Interaction of Cholecystokinin with Plasma Membrane Receptors: Structural Requirements for Activation of Adenylate Cyclase," was selected for presentation at the Annual Meeting of the American Gastroenterological Association on May 23, 1974.

Publications:

Walsh, J. R., and Laster, L.: Enzymatic deamidation of the C-terminal tetrapeptide amide of gastrin by mammalian tissues. Biochem. Med. 8: 432-449, 1973.

Serial No. NIAMDD-DDB-11(c)
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Gastric Emptying

Previous Serial Number: None

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Dr. Andre Dubois, NIAMDD; Dr. Mones Berman, NCI; Mrs. Clementine Sessoms, NIAMDD.

Cooperating Units: Laboratory of Theoretical Biology, NCI

Project Description:

Using a dye dilution technique combined with rapid sampling, gastric emptying and secretion are being evaluated in vivo in man, dogs, and monkeys. Results obtained from such studies are being used to develop a mathematical model of gastric emptying.

Honors and Awards:

Serial No. NIAMDD-DDB-12(c)
1. Digestive Diseases
2. Gastroenterology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cyclic Nucleotide Mediated Functions

Previous Serial Number: NIAMDD-DHDB-6(c)

Principal Investigator: Dr. Jerry D. Gardner

Other Investigators: Drs. Hayden L. Klaeveman and Arnold G. Levy, NIAMDD: Dr. Gerald D. Aurbach, MDB, NIAMDD; Thomas P. Conlon and Trent D. Adams, NIAMDD; Dr. A. J. Tousimis, Biodynamics Research Corporation.

Cooperating Units: Metabolic Diseases Branch, NIAMDD
Biodynamics Research Corporation (Contract #NO1-AM-3-3302)

Man Years:

Total:	2.25
Professional:	.75
Other:	1.5

Project Description:

In vitro systems are being used to characterize the mechanisms by which cyclic nucleotides alter cell function and to explore the mechanism of action of agents whose effect on cell function is mediated by cellular accumulation of cyclic nucleotides. In turkey erythrocytes β-adrenergic catecholamines increase cellular cyclic AMP which in turn stimulates membrane transport of sodium and potassium. In isolated lymphocytes from rat thymus prostaglandin E_1 and cholera toxin increase cellular cyclic AMP which then stimulates membrane transport of several neutral acids. Several gastrointestinal hormones activate adenylate cyclase in plasma membranes prepared from isolated pancreatic acinar cells and also alter various cellular functions; however, at present, it is not clear which, if any, of these functional alterations are mediated by cyclic AMP.

Honors and Awards:

A paper entitled "Mechanism of Action of Cholera Toxin: Receptor Binding and Activation of Adenylate Cyclase," was selected for presentation at the National Meeting of the American Federation for Clinical Research on May 4, 1974.

A paper entitled "Cyclic AMP and Ion Transport," was presented by invitation on a panel on "Cyclic AMP and Ions" at the Seventh Annual Winter Conference on Brain Research at Steamboat Springs, Colorado, January 19-26, 1974.

Publications:

Gardner, J. D., Klaeveman, H. L., Bilezikian, J. P., and Aurbach, G. D.: Effect of beta-adrenergic catecholamines on sodium transport in turkey erythrocytes. J. Biol. Chem. 248: 5590-5597, 1973.

Gardner, J. D., Klaeveman, H. L., Bilezikian, J. P., and Aurbach, G. D.: Effects of ouabain on catecholamine-stimulated sodium transport in turkey erythrocytes. J. Biol. Chem. 249: 516-520, 1974.

Boyle, J. M., and Gardner, J. D.: Sequence of events mediating the effect of cholera toxin on rat thymocytes. J. Clin. Invest. 53: 1149-1158, 1974.

Gardner, J. D., Klaeveman, H. L., Bilezikian, J. P., and Aurbach, G. D.: Stimulation of sodium transport in turkey erythrocytes by cyclic AMP. Endocrinology. In press.

Serial No. NIAMDD-DDB-13 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cholesterol and Biliary Lipid Metabolism in Obesity

Previous Serial Number: NIAMDD-MDB-11(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigator: Dr. Lynn J. Bennion

Cooperating Units: None

Man Years:

Total:	1.9
Professional:	0.7
Other:	1.2

Project Description:

Objectives: This study is designed to determine whether obesity is accompanied by changes in bile which might predispose to cholesterol gallstone formation. Obesity is known to result in increased cholesterol synthesis, and if an excessive amount of cholesterol, relative to bile acids and lecithin, were secreted into the bile of obese persons, the likelihood of cholesterol precipitation and stone formation would be enhanced.

Methods Employed: Biliary lipid composition, biliary lipid secretion rates, and bile acid pool size have been determined in obese Indians and caucasians before and after weight reduction. Post-reduction studies are carried out at least 2 weeks after reestablishment of weight maintenance, in order to eliminate effects due to the catabolic state.

Major Findings: Weight reduction resulted in a significant decrease in the molar percent of cholesterol in gallbladder bile (12.4 vs 7.2) and in hepatic bile (8.3 vs 6.0). The biliary secretion rate of cholesterol was significantly less after weight reduction (75 vs 57 mg/hr), but the outputs of bile acid and lecithin were not significantly altered.

Significance: Our results suggest that: 1) obesity is associated with bile having an increased content of cholesterol, relative to the substances in bile which keep cholesterol from precipitating and forming stones. Obesity is therefore likely to predispose to cholesterol gallstone formation.

2) This increased relative content of cholesterol is due to increased secretion of cholesterol into the bile in obesity, rather than to decreased secretion of bile acid and lecithin. 3) Weight reduction results in improvement in bile composition, and therefore may be a useful approach to the prevention of gallstones.

Proposed Course: We intend to extend these observations to more subjects, multiple sampling of bile in each subject, and the determination of bile acid pool size and turnover kinetics.

Honors and Awards: None.

Publications: None.

Serial No. NIAMDD-MDB-14 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of the Effects of Bile Acid Feeding on Biliary Lipids and Cholesterol Metabolism in Non-Indian Subjects

Previous Serial Number: NIAMDD-MDB-12(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Ronald Adler, Lynn Bennion, William Duane

Cooperating Units: None

Man Years:

Total:	1.1
Professional:	0.4
Other:	0.7

Project Description:

Objectives: Studies from other laboratories have shown that oral feeding of chenodeoxycholic acid (CDCA), but not cholic acid (CA), is effective in eliminating or reducing the size of cholesterol gallstones in man. The mechanism of action of CDCA remains unknown, though its administration is associated with a reduced concentration of cholesterol in bile relative to the solubilizing lipids -- bile salts and phospholipids. This effect could be achieved either by reduced biliary secretion of cholesterol or increased secretion of bile acids and/or phospholipids. In view of the reduction of bile acid pool size in patients with gallstones, the latter mechanism has been favored. The objective of the study is to directly examine the effect of CDCA by measurement of the rate of biliary secretion of cholesterol, bile acids, and phospholipids before and after daily administration of this bile acid for one month.

Methods Employed: Before and after one month of low dose (500-1000 mg/day) CDCA ingestion, the following determinations were made in 10 normal volunteers:

1. lipid composition of gallbladder bile.

2. secretion rate of biliary cholesterol, bile acids and lecithin.

3. bile acid pool size.

4. relative proportions of the individual bile acid species present in gallbladder bile.

1. The molar percent of cholesterol in gallbladder bile was significantly lower after CDCA feeding than in the control period.

2. The rate of secretion of cholesterol was lower after CDCA feeding than in the control period.

3. Bile acid pool size did not change significantly.

4. The relative proportion of CDCA, as compared to other bile acids, increased after low dose CDCA feeding.

5. There was no detectable increase in biliary lithocholic acid during low dose CDCA feeding.

6. Cholic acid was still present in significant amounts in gallbladder bile.

Significance: The results of this study on normal volunteers suggest that therapeutically beneficial effects of CDCA feeding can be achieved at doses lower than those currently being employed in clinical trials. They also suggest that the lessened relative cholesterol content of bile does not depend on expansion of the bile acid pool, but rather that it results from diminished cholesterol secretion. The continued presence of cholic acid in bile indicates that the conversion of cholesterol to bile acids is not abolished by low dose CDCA feeding, and makes it less likely that CDCA feeding will result in an accumulation of body cholesterol. The fact that lithocholic acid did not increase in the bile on subjects fed CDCA suggests that the toxic effects of this poorly absorbed metabolite of CDCA may be avoided.

Proposed Course: Studies terminated. Results are being submitted for publication.

Honors and Awards: None.

Publications: None.

Serial No. NIAMDD-DDB-15 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Effect of Diabetic Control on Cholesterol Metabolism

Previous Serial Number: NIAMDD-MDB-15(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigator: Dr. Lynn J. Bennion

Cooperating Units: None

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description:

Work on this project has been temporarily suspended in favor of other projects. Nothing was accomplished between July 1, 1973 and June 30, 1974.

Proposed Course: We propose to make more measurements of bile composition in the controlled and uncontrolled diabetic state, in order to determine whether diabetic control may be important in the prevention of cholelithiasis.

Honors and Awards: None.

Publications: None.

Serial No. NIAMDD-DDB-16 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Critical Micellar Concentration of Human Biliary Mixed Micelles

Previous Serial Number: NIAMDD-MBD-17(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. William C. Duane

Cooperating Units: None

Man Years:

Total:	1.1
Professional:	0.7
Other:	0.4

Project Description:

Objectives: The bile salt-lecithin mixed micelle serves the important function of solubilizing biliary cholesterol; however, formation of this mixed micelle is poorly understood. We have investigated formation of this mixed micelle by studying the intermicellar bile salt concentration (IMBC) in equilibrium with the mixed micelle. Extrapolation of this parameter to zero mixed micellar bile salt allows estimation of the critical micellar concentration (CMC) of the mixed micelle.

Methods: Equilibrium dialysis has been used to measure the IMBC. This technique is applicable because the mixed micelles are large enough to be retained by the dialysis membrane, but the simple bile salt micelles and bile salt monomers, which comprise the IMBC, are small enough to dialyze. Validation of this technique has been obtained by studying the cholate-lecithin mixed micelle for which IMBC values have been obtained by other investigators. The technique has subsequently been applied to whole gallbladder bile and to artificial systems of pure bile salt and lecithin. In both these applications, the relationship of the IMBC to the mixed micelle bile salt/phospholipid ratio (B/P) has been established. In addition the effect of ionic strength and dilution on the IMBC has been studied.

Results: In all systems studied the IMBC was directly proportional to B/P; however, in pure bile salt-lecithin systems, the IMBC for taurocholate-lecithin micelles was much higher than the IMBC for taurochenodeoxycholate-lecithin micelles. Results for whole bile fall in between these two extremes.

Extrapolation of the IMBC to zero B/P yielded critical micelle concentrations in these systems. The mean CMC of five bile samples was about 0.5 mM while the CMC of the taurochenodeoxycholate-lecithin mixed micelle was about 0.6 mM and the CMC of the taurocholate-lecithin mixed micelle was about 3.0 mM. Simple dilution of bile specimens resulted in a decrease in the IMBC and B/P; however, dilution of bile with B/P held constant did not affect the IMBC. In the range of 0.05 to 0.15 mM, ionic strength did not appreciably affect the IMBC.

Significance: The above studies provide some insight into the formation of bile salt-lecithin mixed micelles. Since dilution of bile reduces the amount of bile salt in the mixed micelle, cholesterol solubility should be reduced by dilution. This reduction in cholesterol solubility by dilution has been demonstrated in other laboratories. The fact that the CMC of the taurocholate-lecithin mixed micelle is so much higher than the CMC of the taurochenodeoxycholate-lecithin micelle suggests that taurocholate has considerably less micelle-forming potential than taurochenodeoxycholate. This observation may explain the reduced capacity of taurocholate to solubilize cholesterol and the ability of chenodeoxycholate to dissolve gallstones in man.

Proposed Course: This project is now essentially complete. It is presently being prepared for publication.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-17 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Hepatic HMG CoA Reductase in Genetically Obese Rats

Previous Serial Number: NIAMDD-MDB-18(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Eunice Flock and William Duane

Cooperating Units: Center for Disease Control, Phoenix Laboratories

Man Years:

Total:	0.7
Professional:	0.3
Other:	0.4

Project Description:

HMG CoA reductase is the rate-limiting enzyme in the synthesis of cholesterol and the amount of this enzyme in the liver increases rapidly after feeding. Peak values have been found by others at midnight in rats fed ad lib. The elevated levels of blood cholesterol in genetically obese rats could be due either to increased production of cholesterol or decreased conversion to bile acids.

In a preliminary study of 5 months duration, ending in June 1973, twelve genetically obese rats and twelve of their nonobese siblings were fed chow diet ad lib in a room with a dark period from 4:30 pm to 8:00 am. The rats were killed by decapitation between 10:00 am and 11:00 am and thus presumably during fasting. The livers were assayed for cholesterol content and HMG CoA reductase activity. The concentrations of cholesterol and triglycerides in blood were also measured.

Methods: Cholesterol in liver was measured after preliminary incubation of the liver in alcoholic KOH and extraction of cholesterol with petroleum ether. Cholesterol and triglycerides in blood were measured simultaneously in the Technicon AutoAnalyzer II.

The HMG CoA reductase assay involved separation of liver microsomes, incubation with radioactive HMG CoA and isolation of radioactive mevalonic acid.

Major Findings: The obese rats weighed over twice as much as their nonobese siblings (600 gm ± 15(S.E.) vs 284 ± 10): liver weights were correspondingly greater (23.1 vs 9.3 gm) and also the ratio of liver weight to body weight was greater (3.87 vs 3.32%). Serum cholesterol was 173 mg/100 ml in the obese rat compared to 61 in the nonobese siblings. Serum triglycerides were 1230 mg/100 ml in the obese rats and 149 in the nonobese rats. Concentrations of cholesterol in liver were similar in both groups (2.46 vs 2.39 mg/gm).

No difference was found between the two groups of rats in the pmoles of MEV-^{14}C formed from [3-^{14}C] HMG CoA per min per mg microsomal protein when assays were made of triplicates of suspensions of microsomal pellets from duplicate samples of liver.

The range of values for the HMG CoA reductase in different rats in each group was great: 19-858 pmoles/min/mg in obese rats and 16-562 in nonobese rats. It appeared that each group was a mixture of fasted and fed rats.

Significance: Although no difference was found between obese and nonobese rats in the amount of HMG CoA reductase per mg of microsomal protein the total amount of this enzyme in the very large liver of the obese rat was much greater than in the nonobese rat and thus much more cholesterol could be supplied to blood and bile.

Proposed Course: Additional studies should be made of genetically obese rats and their nonobese siblings using improvements in the assay procedure for HMG CoA reductase and with controlled periods of lighting and feeding. Basal levels of the enzyme should be obtained after a definite period of fasting and peak levels of the enzyme should be measured at 4 or 6 hours after the beginning of the feeding period.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-18 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Alcohol Metabolism in American Indians and Caucasians: A Comparative Study of Rates, Mechanisms and Adaptive Capacity

Previous Serial Number: NIAMDD-MDB-19(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Lynn J. Bennion and Dr. T. K. Li

Cooperating Units: Indiana University Medical Center

Man Years:

Total:	0.9
Professional:	0.3
Other:	0.6

Project Description:

Objectives: Recent studies have shown genetic and racial differences in the rate of alcohol metabolism. This project is intended to examine racial variations in alcohol metabolism in Southwestern American Indians and caucasians.

Methods Employed: The rate of alcohol metabolism is determined by giving an oral dose of ethanol, based on body weight, and following the rate of disappearance of ethanol from the blood during the ensuing 4-1/2 hours. Blood alcohols are measured using gas-solid chromatography.

Major Findings: Approximately 30 caucasians and 30 Indians have been studied to date. No significant difference between caucasians and Indians in the average rate of alcohol metabolism has been found.

When the data are analyzed in terms of history of alcohol intake, both caucasians and Indians show an increase in rate of alcohol metabolism associated with a history of heavy intake.

Significance: These studies do not confirm the earlier report of more rapid metabolism of alcohol in caucasians than in Indians, and therefore cast doubt on the inference that the high rate of alcoholism among Indians may be related to a low rate of clearance of alcohol from the blood.

Proposed Course: More Indian subjects will be studied to ascertain whether tribal differences in alcohol metabolism, suggested by some of our preliminary data, are real. The results of the Indians versus caucasians comparison will be published together with the results of a companion study being carried out in cooperation with Dr. T. K. Li of the Indiana University Medical Center, in which the isoenzyme patterns of liver alcohol dehydrogenase are being compared in Indians versus caucasians.

Honors and Awards: None.

Publications: None.

Serial No. NIAMDD-DDB-19 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Effect of Phenobarbital upon Biliary Lipid Secretion and Metabolism in Normal Subjects

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigator: Dr. Ronald L. Ginsberg

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	0.3
Other:	0.7

Project Description:

Recent studies have shown that, in the primate, phenobarbital, a known potent inducer of liver microsomal enzymes, had the following effects on biliary lipid metabolism and composition: 1) increased bile flow rates 2) increased bile salt and phospholipid secretion with a concommitant decrease in cholesterol secretion by the liver 3) a consequent decreased cholesterol content in bile relative to bile salts and phospholipids 4) augmented bile salt synthesis rate and bile salt pool size in animals with intact enterohepatic circulations 6) enhancement of the maximal bile salt secretion rate in animals with total biliary fistulae.

The present project proposes to study these parameters in normal human volunteers treated with phenobarbital in order to determine the possible efficacy of this drug in the prevention of cholesterol cholelithiasis.

Methods: Normal volunteers are given a tracer dose of radioactively labelled cholic acid, and a total bile acid pool size, catabolic rate of cholic acid, and synthesis of cholic acid are determined from the subsequent fall in specific activity of cholic acid over the next four days. The relative bile composition with respect to cholesterol, bile salts, and phospholipids is also determined. Hourly secretion rates of cholesterol, bile salts, and phospholipids are also determined by the previously described method of Grundy and Metzger. All studies are performed on the same patients before and after the ingestion of phenobarbital for two months at a dose of 3 mg/kg per day; each patient serves as his own control.

Controls will be studied in the same manner with the substitution of a placebo for phenobarbital.

Major Findings: None.

Significance: The establishment of phenobarbital - a widely-used, safe, inexpensive medication - as a useful prophylaxis against cholesterol gallstone disease would have far-reaching implications for those populations with high incidences of cholelithiasis. Positive results might encourage further investigation as to the efficacy of phenobarbital in dissolving already existing gallstones, hence offering an alternative to surgical intervention.

Proposed Course: Eight to ten volunteers will complete the study during the next four to six months.

Honors and Awards: None.

Publications: None.

Serial No. NIAMDD-DDB-20 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: HMG CoA Reductase in Livers of Hamsters during Gallstone Formation

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Eunice Flock and William Duane

Cooperating Units: Center for Disease Control, Phoenix Laboratories

Man Years:

Total:	0.9
Professional:	0.5
Other:	0.4

Project Description:

The development of cholesterol gallstones in young hamsters fed a high-glucose diet deficient in essential fatty acids has been studied for more than 20 years by Dam and many other investigators. That the formation of the gallstones may be due to increased synthesis of cholesterol is suggested by the greater incorporation of ^{14}C acetate into cholesterol in hamsters after intraperitoneal injection in hamsters on the lithogenic diet compared to the rice-starch diet (Jensen and Dam) and by the greater incorporation of radioactive acetate into cholesterol by liver slices of hamsters on the high-glucose diet (Muroya and coworkers).

The purpose of this investigation was to determine the effect of the lithogenic diet on the rate-limiting enzyme for the synthesis of cholesterol, namely HMG CoA reductase.

Methods: Young male hamsters were kept in a room with a dark period from 2:30 am to 2:30 pm with a feeding period of 6 hours from 2:30 am. It is known that in rats the amount of HMG CoA reductase increases dramatically after feeding reaching a peak in about 6 hours. Twenty-four hamsters were studied; half were maintained on the lithogenic diet and half on Purina chow diet.

The hamsters were killed with CO_2 gas from 13 to 57 days after the feeding of these diets were begun and from 6 to 7 hours after the beginning of the last feeding period. The gallbladders were examined for the presence of

gallstones. The livers were assayed for the HMG CoA reductase and the results were expressed as picomoles of radioactive mevalonate formed per min per mg of microsomal protein. Carcass cholesterol was also measured.

Major Findings: Cholesterol gallstones were found in the gallbladders of two hamsters which had been fed the lithogenic diet for 42 and 48 days respectively. One hamster with 6 gallstones had an HMG CoA reductase of 806 pmoles MEV-^{14}C/min/mg microsomal protein. Levels of 281, 1224, and 6195 pmoles/min/mg were found in other hamsters which had been fed the lithogenic diet for 36 to 42 days. The hamster with 10 gallstones was studied after a 24-hour fast instead of a 6-hour feeding period and had an HMG CoA reductase of only 37 pmoles/min/mg.

None of the hamsters on the chow diet developed gallstones. The enzyme levels in two of these hamsters after a 24-hour fast were 30 and 40 pmoles/min/mg. Surprisingly, similar enzyme levels were found in the hamsters which were studied at 6 or 7 hours after feeding. It is possible that the peak of enzyme activity in hamsters on the chow diet might have occurred earlier in hamsters on the chow diet than in those on the lithogenic diet.

No difference was found in the amount of cholesterol per 100 gm of carcass between hamsters on the lithogenic and chow diets. Hamsters on the lithogenic diet weighed less than those on the chow diet but they had a higher ratio of liver weight to body weight (6.1 vs 4.0%).

An attempt was made to repeat this study with 24 more hamsters but an infection spread through this group and necessitated termination of the experiment.

Proposed Course: Although very high levels of enzyme were found in several of the hamsters on the lithogenic diet, comparison with the data from the hamsters on the chow diet was severely limited by the observation that the levels of the enzyme were all in the fasting range. Information on the diurnal cycle of HMG CoA activity in these hamsters is badly needed.

It appears that there is no difference in the activity of this hepatic enzyme in fasting hamsters. It remains to be proven that there is a striking difference in the fed hamsters.

Honors and Awards: None.

Publications: None.

Serial No. NIAMDD-DDB-21 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effect of Fasting on Bile Acid Synthesis, Bile Acid Pool Size, and Biliary Lipid Composition.

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. William C. Duane, Dr. Ronald Ginsberg, and Dr. Lynn Bennion

Cooperating Units: None

Man Years:

Total:	1.0
Professional:	0.3
Other:	0.7

Project Description:

Bile acid and cholesterol metabolism during fasting have been studied in subhuman primates; however, the effects of fasting on sterol metabolism in man remain poorly understood. We have used isotope dilution techniques to measure bile acid synthesis and pool size in the fed state and again after a four-six day fast in six subjects. In addition the lipid composition of gallbladder bile has been examined in these subjects during the fed and the fasted state.

Methods: Isotopic cholic acid was administered orally and gallbladder bile obtained on the four subsequent mornings. After the fourth bile sample, each subject fasted for five-six days. On the evening of the fourth fasting day cholic acid labelled with a different isotope was administered and gallbladder bile obtained the two subsequent mornings. Total bile acid pool size and cholic acid pool size were calculated from the specific activity in bile obtained the first control day and again the first fasting day. This method of pool size determination has been validated previously in this laboratory and is precise to ± 2.6%. Cholic acid synthesis and fractional catabolic rates were calculated by standard methods for the fed state. Calculation of these parameters for the fasting state was achieved by assuming a single pool model for cholic acid and rapid changes in synthesis and fractional catabolic rates at the beginning of the fast. Gallbladder bile lipid composition and individual bile acid composition were measured by standard techniques.

Results: Total bile acid pool and cholic acid pool were either slightly reduced or unchanged by fasting. Cholic acid synthesis was reduced by a factor of three or four in all subjects during fasting. The fractional catabolic rate of cholic acid was reduced by fasting in all subjects. Fasting reduced the molar percent of cholesterol (relative to the solubilizing lipids, bile acids and lecithin) in all but one subject. Individual bile acid composition did not show any consistent change during fasting.

Proposed Course: Investigative work on this project has been completed. The results will be submitted for publication.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-22 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Rapid Determination of Bile Acid Pool Size

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. William C. Duane, Ronald Adler, Lynn Bennion, and Ronald Ginsberg

Cooperating Units: None

Man Years:

Total:	2.1
Professional:	0.7
Other:	1.4

Project Description:

A simplified isotope dilution technique for measurement of total bile acid pool size has been investigated. A known amount of isotopic cholic acid is administered in the evening. The following morning a sample of gallbladder bile is obtained via a duodenal tube. Total bile salt pool is calculated from the specific activity of unseparated bile acids in this single bile sample.

Methods: In fifteen subjects bile acid pool was measured by this technique on two separate occasions. This data allowed calculation of a figure for precision of the method. In thirteen subjects, pool size as measured by this technique was compared to pool size simultaneously measured by the traditional method of Lindstedt. In four subjects the effect of route of isotope administration on the estimation of pool size was examined by simultaneous administration of intravenous ^{3}H-cholic acid and oral ^{14}C-cholic acid.

Results: The mean precision of the simplified method was 2.6% in the fifteen subjects studied. As predicted by the fact that specific activity is not extrapolated to time zero, pool size as measured by the simplified technique averaged 13.7% higher than simultaneous estimates by the Lindstedt method. Pool size estimated by intravenous isotope was less than 5% different than pool size estimated by oral isotope suggesting that the administered isotope was completely mixed with the bile acid pool.

Significance: The simplified method of bile acid pool size determination provides a highly reproducible estimate of the bile acid pool size. Additional advantages of the simplified method are a four- to eight-fold reduction in required isotope and considerable facilitation of analytical procedures. The method is ideal for large-scale investigations of disorders associated with alterations in bile acid pool size.

Proposed Course: The project is now complete. The data have been written up and submitted for publication. No further work on this project is planned.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-23 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Effect of the Menstrual Cycle and Oral Contraceptives upon Biliary Lipid Composition

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Drs. Lynn Bennion and Ronald L. Ginsberg

Cooperating Units: None

Man Years:

Total:	0.7
Professional:	0.3
Other:	0.4

Project Description:

Previous investigations have demonstrated a considerably higher incidence of cholesterol gallstone disease among females as opposed to males in both caucasian and Southwestern American Indian populations. This study was designed to test the hypothesis that higher levels of progesterone and/or estrogen were responsible for a higher cholesterol saturation of bile and hence an increased predisposition to cholesterol cholelithiasis.

Methods: Normal caucasian and Indian volunteers underwent bile sampling via a double-lumen duodenal tube on two successive nights at three different times during their menstrual cycle: during menses, peri-ovulation, and luteal phase. Plasma estrogen and progesterone levels were measured on each day of bile collection. Two male volunteers were used as controls. Bile was analyzed for molar percent of cholesterol, bile acids, and phospholipids. To date six females and two males have had samples taken over one or more months.

Major Findings: No significant differences in the cholesterol saturation of bile were found in any of the subjects when different times in the menstrual cycle (and different levels of progesterone or estrogen) were compared.

Significance: Thus far it appears that increased levels of progesterone and estrogen and any other effects that vary during the menstrual cycle are not correlated with increased biliary lithogenicity. Hence the cause for

an excessive female incidence of cholesterol cholelithiasis is still undetermined, although one obvious etiology appears to have been eliminated.

Proposed Course: Approximately three to four more subjects will be studied hopefully over several cycles before finalizing the above conclusions.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-24 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Measurement of Biliary Lipid Secretion in the Fasting State

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. William C. Duane

Cooperating Units: None

Man Years:

Total:	0.7
Professional:	0.3
Other:	0.4

Project Description:

Objectives: To determine biliary lipid secretion rates in the fasting state. Recent studies from this laboratory have shown that the composition of bile in the fasting state is much more lithogenic than bile from the same person during feeding. However, measurement of biliary lipid secretion rates during fasting has proven difficult because infusion of a marker into the duodenum results in unpredictable stimulation of gallbladder contraction.

Methods Employed: A technique for the measurement of fasting biliary lipid secretion rates has been developed that does not require the infusion of a marker into the duodenum. This technique involves intravenous infusion of Indocyanine Green at a constant, known rate. By periodically sampling duodenal contents for Indocyanine Green, bile salts, cholesterol, and phospholipids, the secretion rates of the three biliary lipids can be calculated. To establish the validity of this approach, we have measured cholesterol secretion during the formula infusion state simultaneously with Indocyanine Green and with duodenal marker infusion. This should enable us to establish the accuracy and reproducibility of the Indocyanine Green measurement of secretion rate as well as investigate the effects of Indocyanine Green on the biliary lipid secretion rates themselves.

Results: About eight subjects have been studied. In two or three subjects cholesterol secretion as measured by Indocyanine Green was the same as cholesterol secretion measured by intraduodenal marker infusion. In three subjects cholesterol secretion as measured by Indocyanine Green was

much higher than cholesterol secretion as measured by intraduodenal marker infusion. This effect was much more pronounced in the first three to four hours of Indocyanine Green infusion than in subsequent hours of the study suggesting prolonged delay in hepatic Indocyanine Green output. In all studies, cholesterol secretion measured by Indocyanine Green was much more variable than cholesterol secretion measured by intraduodenal marker infusion.

Proposed Course: Because of the variability of cholesterol secretion as measured by Indocyanine Green infusion and because of the prolonged delay in Indocyanine Green output by the liver, this project has been discontinued.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-25 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Estimation of Bile Acid Synthesis Rate by Measurement of $^{14}CO_2$ Excretion after Intravenous Cholesterol-26-^{14}C.

Previous Serial Number: NIAMDD-MDB-14(c)

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Ronald Adler

Cooperating Units: None

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description:

Objectives: Little is known of the factors controlling bile acid synthesis in man. Currently used methods to measure bile acid synthesis require steady state conditions for periods of days to weeks and are not applicable to detection of changes in synthesis over a period of hours or days.

Methods: Bile acid synthesis over short intervals of time could be estimated by monitoring the conversion of cholesterol to bile acids by measurement of $^{14}CO_2$ excretion derived from the three carbon fragment cleaved from the side chain of cholesterol labelled at the C-26 position. This three carbon fragment is released as proprionic acid and subsequently converted to CO_2 during metabolism of the proprionic acid.

Major Findings: Data in three subjects suggest a diurnal variation (related to eating) of bile acid synthesis; however, in one subject the administration of glucose appeared to alter $^{14}CO_2$ output. Since glucose does not stimulate gallbladder contraction and since the alteration in $^{14}CO_2$ output occurred so rapidly, this finding suggests that changes in propionic acid metabolism may result in misleading data regarding bile acid synthesis.

Proposed Course: Because of uncertainties about the effect of propionic acid metabolism on data obtained by this technique, and because Dr. Adler has left the NIAMDD, the project has been discontinued.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-DDB-26 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Output of Biliary Lipids in Southwestern American Indians with Cholesterol Gallstones

Previous Serial Number: NIAMDD-MDB-8(c)

Principal Investigator: Dr. Scott M. Grundy

Other Investigators: Drs. Allan L. Metzger, Ronald Adler

Cooperating Units: None

Man Years:
Total: 0
Professional: 0
Other: 0

Project Description: This project has been discontinued.

Honors and Awards: None

Publications:

1. Grundy, S.M., Duane, W.C., Adler, R.D., Aron, J.M., and Metzger, A.L.: Biliary Lipid Outputs in Young Women with Cholesterol Gallstones. Metabolism 23:67-73, 1974.

2. Adler, R.D., Metzger, A.L., and Grundy, S.M.: Biliary Lipids Before and After Cholecystectomy. Gastroenterology (in press).

Serial No. NIAMDD-DDB-27 (c)
1. Digestive Diseases Branch
2. Phoenix Clinical Research Section
3. Phoenix, Az.

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Cholesterol Gallstone Formation by the Method of Cholesterol Balance

Previous Serial Number: NIAMDD-MDB-16(c)

Principal Investigator: Dr. Scott M. Grundy

Other Investigator: Dr. Ronald Adler

Cooperating Units: Rockefeller University

Man Years:

Total:	0
Professional:	0
Other:	0

Project Description: This project has been discontinued.

Honors and Awards: None

Publications: None

CLINICAL ENDOCRINOLOGY BRANCH

A greatly expanded teaching program in clinical endocrinology has been initiated in the past few years, and has gotten into full swing this year. It encompasses the clinical endocrinology and metabolism units in all of the Institutes, and also cooperates with outside institutions. The clinical associates take clinical rotations on each of the endocrine and metabolism units. There are combined-staff endocrine conferences, teaching rounds and didactic lectures, and medical students from schools throughout the country take part in an elective program. The coordinator is Bruce Weintraub, who rejoined CEB as a senior investigator about 1 year ago, and acceptance of the program has been excellent. It is expected to aid in the continued availability of superior applicants for the clinical associateships, now that military service is no longer mandatory.

Dr. Jesse Roth, Chief of the Diabetes Section, received two major awards in June, 1974 - the Lilly Award of the American Diabetes Association and the Ernst Oppenheimer Memorial Award of the Endocrine Society - presented at the annual meetings of these societies.

Staff exchanges with laboratories in Naples, Marseilles and Paris took place, and foreign scientists from Germany, Hungary, India, Israel, Italy, Japan and Switzerland served as full-time fellows or associates in the Branch's laboratories.

I. Thyroid Biochemistry

A. Thyroid Cell Membrane and the Mechanism of TSH Action

The initial step in TSH action on the thyroid cell is binding to receptors on the plasma membrane. This has been studied directly with [^{3}H]TSH and [^{125}I] TSH, revealing two classes of binding sites with $K \simeq 2.5 \times 10^{8} M^{-1}$ and $\simeq 1 \times 10^{6} M^{-1}$. Activators or inhibitors of thyroid adenyl cyclase were studied in relation to TSH binding and were classified in four groups: those which (1) affect cyclase and binding in a parallel fashion (Ca^{2+}, Na^{+}, Li^{+}, cobramine B), (2) enhance cyclase but decrease binding (K^{+}, Mg^{2+}), (3) inhibit cyclase but enhance binding (phospholipase A, filipin), (4) alter cyclase but not binding (ITP, GTP, F^{-}, chlorpromazine, gramicidin B, valinomycin). It was concluded that most modulators of thyroid cyclase act at a stage beyond TSH binding and that the relationship between binding and cyclase activation is complex (Wolff, Moore).

One possible mechanism for TSH action is through activation of cyclic AMP-dependent protein kinases. The soluble protein kinase from bovine thyroid has been purified and characterized. In addition, kinase activity has been demonstrated in thyroid plasma membrane fractions. This membrane-bound kinase shows cAMP-dependent activity with exogenous substrates (e.g., histone) but does not require cAMP to act on protein within the membrane fraction itself (McClung, Rall).

B. Iodoproteins

Studies on the quarternary structure of thyroglobulin (TG) have progressed through the use of the guinea pig. Whereas hog, beef, rat and human TG showed considerable size heterogeneity after reduction and alkylation, guinea pig TG showed only 3 predominant bands with apparent molecular weights (SDS gel electrophoresis or sedimentation equilibrium) of 330,000, 250,000 and 155,000 or 290,000, 200,000 and 105,000, depending on the method used. Chemical studies to further define these subunits are in progress (Bilstad, Haberli, Rall, Edelhoch).

Prior studies showed that sialic acid incorporation occurred at a late stage in thyroglobulin synthesis, and that its absence in a thyroid tumor was associated with defective thyroglobulin secretion. It has now been demonstrated by studies with labeled carbohydrates, amino acid and iodine that sialic acid addition occurs entirely within the thyroid cell. It has also been shown that most of the iodine incorporation occurs on newly synthesized thyroglobulin, although the site of addition may be in the follicle lumen at the apical cell border (Monaco, Robbins).

C. Thyroid Hormone Secretion

Tubulin functions in the initiation of thyroid hormone secretion - i.e., in endocytosis of thyroid colloid. Thyroid tubulin has now been purified and found to resemble tubulins of cilia, flagella and brain, although some amino acid differences were demonstrated. The endocytosis inhibitor, colchicine, develops marked fluorescence when it combines with tubulin and this provides a convenient means to study colchicine binding (Bhattacharyya, Wolff).

D. Control of Thyroid Function

The regulation of TSH production by thyrotropin releasing hormone (TRH) in women has been investigated further. It was reported earlier that postpartum suckling causes prolactin in serum to rise without increasing TSH. It has now been shown that this dissociation in pituitary secretion is not due to increased free or total thyroid hormone levels in the blood.

Further information on the regulation of TSH subunit production has been obtained by studying the effects of thyroid hormone feedback on pituitary hypersecretion in myxedema. Secretion of TSHβ is decreased more than TSHα, suggesting that the former is rate-limiting with respect to TSH production

The correspondence between suppression of TSH secretion and the clinical state of hyperthyroidism has been examined in patients with hyperfunctioning thyroid nodules. Even though clinically and chemically euthyroid, these patients show a slight fall in serum hormone levels associated with increased TSH secretion after the nodules are removed. This indicates that TSH suppression is not necessarily indicative of hyperthyroidism (Weintraub).

E. Measurement of Iodocompounds

A novel technique for iodine analysis which does not require chemical

determination has been devised. It consists of (1) conversion of the iodine to a single oxidation state, (2) addition of a trace of radioactive iodine in the same state, (3) stoichiometric reaction with a second labeled element. Analysis of isotope ratio and recoveries permits calculation of iodine content of the sample (Lewallen).

F. Thyroxine-Protein Interactions

Refined techniques for the detection of low affinity thyroxine-transport proteins in serum have been developed. These are based on the change in electrophoretic mobility of the complex compared to the uncomplexed protein. It has been demonstrated that a portion of the γ globulin, β lipoprotein, α_1-lipoprotein and two pre-β lipoproteins can all bind thyroxine, and thus may play a role in thyroid hormone physiology (Lewallen).

The interaction of thyroxine-binding prealbumin (PA) with thyroxine (T_4) was difficult to understand fully because there appeared to be a single binding site for T_4 and for certain T_4 analogs, but 2 equivalent sites for other T_4 analogs, ANS and DNS-glycine. Further studies with dialysis of [^{125}I] T_4 and [^{3}H]ANS showed that there were in fact 2 sites for T_4. Taken together with x-ray diffraction analysis of PA crystals these findings strongly suggest a negatively cooperative ligand-ligand interaction between bound T_4 molecules (Ferguson, Cahnmann, Edelhoch, Robbins).

II. Polypeptide Hormones

A. Structure

The ANS fluorescence assay previously developed for human luteinizing hormone (hLH) and chorionic gonadotropin (hCG) has been applied to bovine thyrotropin (bTSH). With all three hormones, dimerization is induced by ANS, and binding to the dimer is preferential. There are 3-4 moles of ANS bound per molecule of hCG, exhibiting positive cooperativity. In the case of hLH, 1 molecule of ANS is bound per dimer and exhibits negative cooperativity (Ingham, Edelhoch).

B. Circulating Forms of Polypeptide Hormones

In a variety of patients after TRF (e.g., subjects during pregnancy or lactation, and in patients with chromophobe adenoma and galactorrhea, circulating prolactin behaved like monomeric pituitary prolactin on gel filtration. In one subject with acromegaly, on the other hand, 70% of the plasma immunoreactive prolactin was of apparently large molecular size. Thus prolactin in plasma can exhibit the heterogeneity found with many other polypeptide hormones (Weintraub, Rosen). In other patients with acromegaly, about 25% of plasma immunoreactive growth hormone was found in the void volume of Sephadex G-100 columns, and therefore differed from pituitary GH as well as from the previously described "big" GH form (Rosen, Diamond).

C. Ectopic Protein Production by Tumors

Unbalanced production of chorionic gonadotropin subunits (hCG-α and hCG-β)

were previously detected in a pancreatic adenocarcinoma, gastric carcinoid and bronchogenic carcinoma. A prospective study of 123 patients with bronchogenic carcinoma yielded 7 new cases of ectopic placental protein production including placental lactogen and placental alkaline phosphatase as well as hCG.

In two patients, tumor was removed and maintained in cell culture for up to 3 years. Clonal strains derived from these tumors produce different amounts of hCG, hCG-α or hCG-β. In the gastric carcinoid cells, an unusual α-like subunit was found. In addition to a lower apparent molecular weight than authentic hCG-α, this material failed to combine in vitro with hCG-β, a phenomenon which might affect the ability of the tumor to secrete hCG (Rosen, Weintraub).

D. Control of Polypeptide Hormone Secretion

The response to synthetic gonadotropin releasing factor (LRF) in 12 patients with Kallman's syndrome (congenital anosmia and hypogonadism) was studied. Reponses varied from trivial release to excess release of LH, FSH or both (Rosen, Weintraub).

E. Insulin Receptors: Demonstration of Site-Site Interaction.

Under steady state conditions, growth hormone bindings with receptor sites appear to show a single order of sites. As expected, the dissociation rate of ^{125}I growth hormone from its receptor sites is the same whether dissociation is produced by dilution or by dilution in the presence of an excess of unlabeled hormone. By contrast, at steady state, insulin demonstrates a curvilinear Scatchard plot; the majority of peptide hormone receptors demonstrate this curvilinear relationship. Dilution alone induces a much slower dissociation rate than dilution in the presence of an excess of cold insulin, indicating that occupancy of the empty receptor sites by unlabeled hormone produces a decrease in affinity of all of the receptor sites for insulin. Induction of this negative cooperative interaction is a characteristic of all biologically active insulin analogues, except for a few. Analogues that are missing the C terminal amino acid of the A chain or any of several amino acids on the C terminal region of the B chain, do not induce negative cooperative interactions although they bind to the biologically important insulin receptor site and produce the typical biologic action of insulin. Thus the region of the insulin molecule that is responsible for these negative cooperative interactions can be precisely localized. A large number of other studies have been performed using this approach, which has produced a much more unified picture of hormone receptor-receptor interactions (De Meyts, Roth).

F. Regulation of Insulin Receptors In Vivo and In Vitro

An inverse relationship between the chronic level of circulating insulin in vivo and the number of insulin receptor sites per cell has been found. Hypoinsulinemic states such as prolonged fasting, adrenalectomy, or hypophysectomy are characterized by an increase above normal in the number of insulin receptor sites per cell. Conversely, obesity (both genetic and acquired forms in rodents and in man), glucocorticoid excess, and a rare syndrome of extreme insulin resistance of adolescent females, which are characterized by hyper-

insulinemia, have been shown to be associated with a deficiency of insulin receptor sites per cell. The insulin receptor sites that remain are normal by a variety of sensitive criteria. Control of the hyperinsulinemia is associated with the restoration of the receptor concentrations per cell toward normal. In all cases, acute changes in plasma insulin were without effect (Soll, Kahn, Roth, Goldfine). The effect of chronic hyperinsulinemia on the insulin receptor concentration per cell could be reproduced in vitro by the addition of insulin to cultured human lymphocytes. The time of onset and the magnitude of the loss of receptors was related to the magnitude of the insulin concentration to which the cells are being exposed. Removal of the insulin from the medium resulted in a restoration of the receptors over 24 hours. Addition of protein synthetic inhibitors caused a decrease in both the loss of receptors and in their restoration (Gavin, Lesniak, Roth).

In further studies of insulin receptors on "peripheral lymphocytes" it has been found that the major cell types in the mononuclear cell preparations from peripheral blood (Boyum "lymphocytes") that bind insulin is the monocyte. The lymphocytes themselves bind much less insulin per cell (Bianco, Kahn).

G. NSILA-s (Nonsuppressible Insulin-like Activity, Soluble in Acid Ethanol

In collaboration with scientists at the University of Zurich, we have shown that the circulating insulin-like peptide, NSILA-s, reacts with insulin receptors in both liver and in lymphocytes in direct proportion to its insulin-like potencies. In addition, it is fully effective in the negative cooperative interactions of the insulin receptor and in regulation of the insulin receptor of lymphocytes in vitro. It also inhibits the degradation of ^{125}I-insulin by the insulin degrading proteases of hepatic plasma membrane. Thus, except for immunoreactivity, this molecule is remarkably insulin-like. Using NSILA-s that had been labeled with ^{125}I, we demonstrated another set of receptors in liver that bind NSILA-s. This receptor binds NSILA-s much more strongly than it does bind proinsulin or insulin. For the radioreceptor assay of NSILA-s in plasma, plasma was filtered on G-50 Sephadex in acetic acid, and the effluent fractions were neutralized and assayed by this radioreceptor assay. Patients with extrapancreatic tumors in association with hypoglycemia were found to have elevated circulating levels of plasma NSILA-s. Thus far these elevations appear to be specific for patients with this syndrome (Megyesi, Kahn, Gorden, Roth).

H. Studies of Acromegaly

The study of pituitary irradiation for acromegaly is beginning its 10th year of followup. The results of the five-year followup were presented and published in a new volume that reviews pituitary gland tumors (Gorden, Roth).

A prospective study of cardiovascular disease in acromegaly was completed and accepted for publication. Another study on other aspects of visceromegaly in acromegaly have also been accepted for publication. Both these studies show that although cardiomegaly, hepatomegaly, and splenomegaly are very common at postmortem examination, their detection by clinical

maneuvers in vivo indicate a second disease in addition to acromegaly (McGuffin, Gorden, Roth, Sober).

I. Growth Hormone Receptors

A large number of human growth hormone preparations were assayed by in vivo bioassay, by radioreceptor assay (RRA), and by radioimmunoassay (RIA). The correlation between the bioassay and the radioreceptor assay were excellent while the correlation between the radioreceptor assay and the immunoassay were not as close. Plasma and pituitary growth hormone preparations were filtered on Sephadex G-100 and the effluent fractions were assayed by both immunoassay and radioreceptor assay. It was found that the big growth hormone component had markedly reduced radioreceptor activity, which ranged from 0-20% of its immunological activity, and probably accounts for divergences between RRA and RIA (Lesniak, Gorden, Roth).

The human growth hormone receptor of the cultured lymphocyte could be solubilized in the absence of detergent by incubating the cells at high density in a low protein mileu (McGuffin, Gavin, Gorden, Roth).

Growth hormone regulated the concentration of growth hormone receptor sites per cell by a direct effect on the cell. The cells are exquisitely sensitive to regulation by growth hormone so that at 2 ng/ml, a low resting level, which occupies only 10% of the receptor sites, produced a 30-50% loss of receptor sites per cell over a period of 18-24 hours. Following removal of growth hormone from the medium, the receptor sites reappeared with more than half of them restored by six hours and all of them restored by 24 hours. Inhibition of new protein synthesis interferred with the reappearance of the receptors (Lesniak, Roth, Gavin).

III. Corticosteroid Secretion

Prevous work showed that corticosteroid secretion by adrenal tumor cells in culture responded to colchicine in a manner similar to their response to ACTH except that it occurred beyond the step of cAMP generation. It has now been shown that choleragen has a similar effect at low concentration although it also stimulates adenyl cyclase at a 30-fold higher concentration. Another adrenal cell line which lacks a functional ACTH receptor also was able to respond to both colchicine and choleragen (Wolff, Temple).

IV. Protein Structure

The molecular properties of ApoA-II, the second most abundant apoprotein in the high density lipoprotein (HDL) of human serum have been characterized. This protein has a relatively high helical content and considerable tertiary structure. By analogy with ApoA-I, it is thought that the protein stability is enhanced in the lipid matrix of HDL as compared to the lipid-free state. Lysolethicin binds to ApoA-II with an affinity of 10^7, and affects the protein structure (Edelhoch, Gwynne).

A zinc-containing protein has been purified from parotid saliva and may be necessary for taste acuity. Its biological properties and clinical correlations are under study (Edelhoch).

V. DNA Synthesis and Fibroblasts

Non-suppressible insulin-like activity (NSILA-s) stimulated DNA synthesis in cultured human fibroblasts at concentrations present in human serum. The stimulation observed with maximally effective concentrations of NSILA-s and insulin was not additive, suggesting that NSILA-s and insulin may show a common mechanism of action (Rechler, Wells, Podskalny).

Serial No. NIAMDD/CEB 1c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Nonenzymic Synthesis of Thyroxine and Thyroxine Analogs

Previous Serial Number: Same

Principal Investigator: H. J. Cahnmann

Other Investigators: None

Cooperating Units: A. Nishinaga, Ph.D.
Department of Synthetic Chemistry
Faculty of Engineering
Kyoto University
Kyoto, Japan

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 2c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Thyroxine-Protein Interactions

Previous Serial Number: Same

Principal Investigators: R. N. Ferguson, Ph.D., H. Edelhoch, Ph.D., H. J. Cahnmann, Ph.D., J. Robbins, M.D.

Other Investigators: M. Gershengorn, M.D., R. Lord, Ph.D., M. Brackbill, H. Saroff

Cooperating Units: Laboratory of Biophysical Chemistry, NIAMDD

Man Years:

Total: 5 1/4
Professional: 3 3/4
Other: 1 1/2

Project Description:

The interaction of thyroxine-binding prealbumin (PA) with thyroxine, compounds structurally related to thyroxine, and certain fluorescent dyes was difficult to understand fully because there appeared to be a single binding site for thyroxine and certain analogs, but 2 equivalent sites for other analogs as well as for 8-anilino-1-naphthaline sulfonate (ANS) and 5-dimethylaminonaphthaline-1-sulfonyl (DNS)-glycine. Further studies to characterize the interactions were done by dialysis with [^{125}I]thyroxine and [^{3}H]ANS. For this purpose, a method for preparing [^{3}H]ANS by catalytic exchange with $^{3}H_2O$ was developed.

These studies showed that there are, in fact, two binding sites for thyroxine, but that the second site has an apparent affinity which is approximately two orders of magnitude lower than the first site. Models were developed for competitive binding to T_4 and ANS to two independent sites or two interacting sites. The apparent binding constants for independent sites are: for T_4, $K_1 = 1.0 \times 10^8 M^{-1}$, $K_2 = 9.5 \times 10^5 M^{-1}$; for ANS, $K_1 = 9.5 \times 10^5 M^{-1}$, $K_2 = 2.1 \times 10^5 M^{-1}$. Although both models fit the data equally well, the x-ray diffraction studies of PA, which indicate 2-fold molecular symmetry in the binding region, enabled us to conclude that binding of T_4 occurs with negative ligand-ligand interaction. ANS demonstrated little or no interaction. This is one of the first demonstrations of this type of ligand-ligand interaction.

Studies were also done with DNS covalently coupled to PA. These showed directly that DNS occupied the T_4-binding sites on the protein and forms a basis for affinity-labeling of the binding sites.

The major thyroxine transport protein of human serum, thyroxine-binding globulin (TBG), has been purified by affinity chromatography utilizing thyroxine-Sepharose columns followed by ion exchange chromatography and gel filtration. Work has begun on characterizing the molecular and binding properties.

Honors and Awards: None

Publications:

1. Pages, R. A., Robbins, J. and Edelhoch, H.: Binding of thyroxine and thyroxine analogs to human serum prealbumin. Biochemistry 12: 2773-2779, 1973.

2. van Jaarsveld, P., Branch, W. T., Robbins, J., Morgan, F. J., Kanda, Y., and Canfield, R. E.: Polymorphism of Rhesus monkey serum prealbumin: purification and partial structure. J. Biol. Chem. 248: 7898-7903, 1973.

3. van Jaarsveld, P. P., Branch, W. T., Edelhoch, H. and Robbins, J.: Polymorphism of Rhesus monkey serum prealbumin: molecular properties and binding of thyroxine and retinol-binding protein. J. Biol. Chem. 248: 4706-4712, 1973.

4. van Jaarsveld, P. P., Edelhoch, H., Goodman, D. S. and Robbins, J.: The interaction of human plasma retinol-binding protein with prealbumin. J. Biol. Chem. 248: 4698-4705, 1973.

Serial No. NIAMDD/CEB 3c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Analytical Procedures for Qualitative and Quantitative Determination of Iodoamino Acid

Previous Serial Number: Same

Principal Investigator: H. J. Cahnmann, Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Work has progressed on the chromatography of iodoamino acid using small cation exchange resins. Using gradient elution, it is possible to separate mono- and diiodo-tyrosine, triiodothyronine, thyroxine and mono- and diiodohistidine in a single column run.

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 4c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1973 through June 30, 1974

Project Title: Structure of Polypeptide and Protein Hormones

Previous Serial Number: Same

Principal Investigators: H. Edelhoch, Ph.D., J. Gwynne, M.D., K. Ingham, Ph.D., G. Palumbo, Ph.D.

Other Investigators: R. Pollet, M.D., R. Henkin, M.D., B. Brewer, M.D. R. E. Lippoldt

Cooperating Units: Hypertension-Endocrine Branch, NHLI; Molecular Disease Branch, NHLI

Man Years:

Total: 5 3/4
Professional: 4 1/2
Other: 1 1/4

Project Description:

The ANS fluorescence assay developed previously for human luteinizing hormone (hLH) and human chorionic gonadotropin (hCG) has been extended to bovine thyrotropin (bTSH). The rates of dissociation and recombination of the subunits of bTSH have been measured under various conditions, using ANS fluorescence as well as UV difference spectroscopy. The interaction of ANS with hLH, hCG, and bTSH results in a ligand-induced dimerization of these hormones. The cooperative nature of the ANS binding curves has been explained on the basis of the preferential binding of ANS to the dimer form. hCG binds 3-4 moles of ANS per mole of dimer and exhibits positive cooperativity whereas hLH binds 1 molecule of ANS per dimer and exhibits negative cooperativity.

The molecular properties of apoA-II (M = 17,000), the second most abundant apoprotein in HDL, have been characterized by spectroscopic and hydrodynamic techniques. It has a relatively high helical content and considerable tertiary structure. The single disulfide bond, which joins two identical polypeptide chains, was found to contribute significantly to the stability of the secondary and tertiary structure.

The interaction of apoA-II with the phospholipid, lysolecithin, was also studied. A binding affinity of about 10^7 was observed. The effect of binding on the conformation of apoA-II was determined by circular dichroism and polarization of fluorescence measurements.

The behavior of the apoproteins of human HDL was compared in their free state with that in the native form of the lipoprotein. ApoA-I could be analyzed for independently since it contains tryptophan whereas apoA-II does not. It was found that apoA-I is much more stable in the lipid matrix of HDL than when in its lipid-free state.

The denaturation of thyroglobulin has been investigated in dilute acid solutions. The rates could be followed by fluorescence since the concentration of protein could be kept very low where aggregation is avoided. The pH dependence of the denaturation was evaluated by fluorescence and adsorption measurements.

A zinc-containing protein, which is necessary for taste acuity, has been purified 5000-fold from parotid saliva. It has a molecular weight of 37,000 and no subunits. Its biological and clinical properties are under investigation.

Honors and Awards: None

Publications:

1. Aloj, S. M., Edelhoch, H., Ingham, K. C., Morgan, F. J. and Canfield, R. D.: The Rates of Dissociation and Recombination of the Subunits of Chorionic Gonadotropin. Arch. Biochem. Biophys. 159: 497, 1973.

2. Ingham, K. C., Aloj, S. M. and Edelhoch, H.: The Rates of Dissociation and Recombination of the Subunits of Human Luteinizing Hormone. Arch. Biochem. Biophys. 159: 596, 1973.

3. Ingham, K. C., Aloj, S. M. and Edelhoch, H.: The Rates of Dissociation and Recombination of the Subunits of Bovine Thyrotropin, in press.

Serial No. NIAMDD/CEB 5c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Measurement of Iodocompounds in Biological Materials

Previous Serial Number: Same

Principal Investigator: C. G. Lewallen, M.D.

Other Investigators: H. Hoch, Ph.D.

Cooperating Units: Veterans Administration Center, Martinsburg, West Virginia

Man Years:

Total: 1
Professional: 1
Other: 0

Project Description:

Investigative work has for the most part been directed along the following lines:

1) We have continued the study of thyroxine (T_4) binding to human serum proteins utilizing zonal electrophoresis in agarose gel. The conventional technique is capable of demonstrating thyroxine binding by only 3 protein carriers: T_4-binding prealbumin (TBPA), T_4-binding α-globulin (TBG) and albumin. Last year we reported the development and application of 2 novel electrophoresis techniques of considerably greater sensitivity than the conventional technique. In the first, (post electrophoresis labeling), labeled T_4 is applied to the gel (in a manner analogous to dye staining) after electrophoretic resolution of the proteins. This simple and sensitive technique is capable of regularly demonstrating T_4 binding by 5 serum proteins: TBPA, albumin, TBG, α_1-lipoprotein, and β-lipoprotein. The second technique (mobility method) involves comparisons of measured protein electrophoretic mobilities in gels in which carrier T_4 has, or has not, been incorporated. An increase in protein anodic mobility in the presence of T_4 suggests binding of anionic T_4. This technique regularly shows T_4 binding by α-lipoprotein and albumin. It is noteworthy that both techniques circumvent the dissociation of T_4 from weak binders during electrophoresis which may be one reason for the lesser sensitivity of the conventional technique.

This year we have extended studies of the mobility method. From some theoretical considerations it appeared that the mobility method should be

more sensitive than post electrophoresis labeling but experimentally in our hands this did not at first prove to be the case (see above). Detection of differences in mobility on separate gel runs admittedly requires tedious measurements and careful control of conditions even when mobility reference substances are used. We have instituted 2 changes which have markedly simplified the technique and improved its sensitivity for demonstrating T_4 binding. (a) The control and experimental runs are carried out simultaneously on the same glass plate. The plate initially is "divided" into 3 lanes, the central one is "masked" off, and gel formed over the 2 edge lanes. The mask is then removed and gel containing carrier T_4 is formed in the central lane. The single origin well near the cathodal end of the plate is common to all 3 lanes. Electrophoresis of serum, fixation, and staining are by standard methods. Labeled T_4 and autoradiography are not required. A protein component not exhibiting a change in mobility from T_4 binding appears on the stained plate as a continuous straight zone extending across all 3 lanes. A component showing binding exhibits an anodic bend in the central lane readily detectable by its continuity with its more cathodic corresponding straight portions in the 2 edge lanes. No measurements of mobility are required. (b) If several runs on the same serum are stained by different dyes, it is sometimes possible to show a mobility change in a component which is not completely resolved electrophoretically from an overlying component not showing a change. Runs on normal human serum stained with Coomassie Blue show T_4 binding by β-lipoprotein, albumin and the most cathodic portion of γ-globulin. Gels stained with the lipid stain Oil Red show T_4 binding by β-lipoprotein, α_1-lipoprotein, and 2 pre-β-lipoproteins.

It seems likely that the T_4 binding proteins other than TBPA, TBG and albumin demonstrated by these new techniques have weaker binding affinities for T_4. The biological role of weak T_4 binders awaits further study. It is not unlikely that their rates of T_4 dissociation are high and could thus be important in the very rapid exchange of T_4 that occurs between plasma and liver. Another possible role is in specific tissue uptake of T_4.

2) Despite the newer techniques for T_3 and T_4 quantitation the need persists for a simple iodine quantitative method comparable to the cerate-arsenite method in sensitivity but without the latter's well-known shortcomings. The literature has been searched extensively and has little to offer. We have done some preliminary work using what appears to be a novel and promising approach. The iodine unknown is brought to a single oxidation state (say I^-) and carrier free radioactive label added to permit calculation of recovery. A suitable electrophoretic support is chosen and 2 origin wells are formed, one near the anodic and one near the cathodic end. The sample (if I^-) is placed in the cathodic well. In the opposite well is placed a labeled complexing or precipitating agent of known specific activity carrying an opposite charge (say Ag^+) and chosen such that it can be differentially counted in the presence of the iodine label. Electrophoresis results in a collision course with formation of an immobile precipitate or complex somewhere between the two wells. Hopefully, the reactant in excess will migrate beyond the product zone leaving a pure product zone of known stoichiometry. The product zone is removed and the 2 labels counted. Counts of the precipitating agent, its known specific

activity, and the known stoichiometry of the product permits calculation of the iodine in the product zone. This, together with iodine recovery computed from iodine label in the product zone permits calculation of the iodine content of the sample.

In preliminary runs the product zones have been exceedingly sharp and well defined.

3) The effects of temperature on the plasma concentratoin of free T_4 and on the rates of dissociation of T_4 from plasma proteins have been reported in the literature. In addition the distribution of labeled T_4 among the serum protein carriers after electrophoresis in cooled gels has been reported in at least one paper (Davis and Gregerman, J. Clin. Endo. Metab. 30: 237, 1970). However, we are notaware of any reports of electrophoretic studies in which the temperature has been measured in the electrophoretic support during electrophoresis and the effects of temperature on T_4 distribution pattern assessed. To attack this problem we have designed and constructed an apparatus which has been demonstrated to be capable of preventing fluctuations in temperature of more than about 1°C in thin agarose gels during electrophoresis. Temperature has been measured with a small thermistor embedded in the gel. A systematic study of temperature effects on T_4 distribution patterns is underway.

Honors and Awards: None

Publications:

1. Hoch, H. and Lewallen, C. G.: Low Affinity Binding of Thyroxine to Proteins of Human Serum. J. Clin. Endo. Metab. 38: 663-673, 1974.

Serial No. NIAMDD/CEB 6c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Thyroid Iodoproteins

Previous Serial Number: CEB 21c

Principal Investigators: J. Bilstad, M.D., A. Haberli, Ph.D.

Other Investigators: J. E. Rall, M.D., Ph.D., H. Edelhoch, Ph.D.

Cooperating Units: Office of the Director, NIAMDD

Man Years:

Total: 2 3/4
Professional: 2 1/4
Other: 1/2

Project Description:

The goal of this project is to determine the number, size and chemical composition of the primary polypeptide chains of the major thyroid iodoprotein, 19 S thyroglobulin (MW 660,000).

In the presence of dissociating agents normally iodinated 19 S partially dissociates into a 12 S species (MW 330,000), the smallest noncovalently bound subunit. Following complete reduction and alkylation, hog, beef, rat and human thyroglobulin showed considerable size heterogeneity with still 12 S being the predominant band, when analyzed by SDS gel electrophoresis. In addition to the 12 S band there are approximately 10 bands with molecular weights between 10,000 and 300,000.

In contrast to the species mentioned above, guinea pig 19 S thyroglobulin, after reduction and alkylation, showed 3 major bands with remaining 12 S (now called A), B (MW 250,000) and C (MW 155,000). These molecular weights were determined by SDS gel electrophoresis. The distribution among these bands was about 38% for A, 18% for B and 42% for C, as determined by densitometry of stained gels. Attempts were made to isolate these 3 units by column chromatography in different concentrations of guanidine-HCl, of which the 6 M guanidine-HCl Agarose A 15 M column showed the best separation. Further experiments with the same Agarose column (A 15 M), but equilibrated with 0.1% SDS-phosphate buffer, showed similar results, namely a partial separation of these 3 major units. The 3 major pools were rechromatographed on the same columns, and

by making narrow cuts 95% pure pools could be obtained. Sedimentation equilibrium centrifugation in 6 M guanidine-HCl gave the following molecular weights: 290,000 for A, 200,000 for B and 105,000 for C. These results are not in a close accordancy with the ones from gel electrophoresis, but the difference may be in part due to the uncertainty of the partial specific volume.

Further chemical studies such as amino acid composition (including iodo-amino acids) and sugar composition are in progess.

Honors and Awards: None

Publications:

1. Bilstad, J., Edelhoch, H., Lippoldt, R. E., Rall, J. E. and Salvatore, G.: Isolation and Characterization of Discrete Fragments of 27 S Thyroid Iodoprotein. Arch. Biochem. Biophys. 151: 341-349, 1972.

2. Frati, L., Bilstad, J., Edelhoch, H., Rall, J. E. and Salvatore, G.: Biosynthesis of the 27 S Thyroid Iodoprotein. Arch. Biochem. Biophys., 1974, in press.

Serial No. NIAMDD/CEB 7c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Protein Synthesis in the Thyroid Gland

Previous Serial Number: CEB 8c

Principal Investigator: J. Robbins, M.D.

Other Investigators: Motomori Izumi, M.D., F. Monaco, M.D., P. De Nayer, M.D.

Cooperating Units: University of Rome, Rome, Italy; University of Louvain, Louvain, Belgium

Man Years:

Total: 1 1/4
Professional: 1 1/4
Other: 0

Project Description:

Prior studies showed that sialic acid incorporation into thyroglobulin occurred at a late stage in the synthesis of this protein. In a rat thyroid tumor which was deficient in sialyl transferase activity, thyroglobulin secretion was defective. Another late event in thyroglobulin synthesis is iodination to form iodotyrosines and thyroid hormones, but this appears to take place after secretion from the thyroid cell into the colloid. The site of sialic acid incorporation was, therefore, investigated by a kinetic method and by the use of a protein-synthesis inhibitor, cycloheximide. Labeled precursors for carbohydrates, and labeled amino acid and iodine, were used. It was shown that sialic acid addition occurs entirely within the thyroid cell. Iodine incorporation was also shown to occur mainly on newly synthesized thyroglobulin, but may occur in the follicle lumen at the apical border of the thyroid cell.

Honors and Awards: Honorary Fellow, Belgian American Educational Foundation, May - June, 1973

Publications: None

Serial No. NIAMDD/CEB 8c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies in Thyroid Disease

Previous Serial Number: CEB 9c

Principal Investigators: J. Robbins, M.D., M. Gershengorn, M.D., B. D. Weintraub, M.D.

Other Investigators: Motomori Izumi, M.D.

Cooperating Units: None

Man Years:

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

In the treatment of patients with functioning carcinoma of the thyroid with radioactive iodine, the goal is to give the maximum possible radiation to the tumor while avoiding dangerous radiation to other tissues, especially the bone marrow. Our earlier studies with lithium carbonate in hyperthyroidism are being applied to patients with thyroid carcinoma, since this drug slows the thyroid iodine secretion rate and therefore may produce the desired effect with respect to differential radiation exposure. In one patient studied thus far, lithium reduced the iodine secretion rate from bone metastases of follicular thyroid carcinoma.

Honors and Awards: None

Publications:

1. Robbins, J., Rall, J. E. and Gorden, P.: The Thyroid and Iodine Metabolism. In Bondy, P. and Rosenberg, L. (eds.): Duncan's Diseases of Metabolism. Philadelphia, PA, Saunders, 1974, p. 1009.

2. Robbins, J.: Thyroiditis. Conn, H. (ed.): Current Therapy. Philadelphia, PA, Saunders, 1973, p. 471.

3. Robbins, J. and Rall, J. E.: Clinical Evaluation of Hormonal Excess and Deficiency. In Peptide Hormones. Amsterdam, North-Holland Publishing Co., 1973, pp. 498-507.

4. Robbins, J.: Radioassay and the Thyroid Gland. Metabolism 22, 1973.

5. Carlson, H. E., Robbins, J. and Murphy, D. L.: The Effect of Lithium on Thyroid Iodine Release in Patients with Primary Affectve Disorder. Psychopharmacologia 35: 249-256, 1974.

6. Robbins, J.: Inherited Variations in Thyroxine Transport. Mount Sinai J. Med. 40: 511-519, 1973.

Serial No. NIAMDD/CEB 9c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Ectopic Protein Production by Tumors

Previous Serial Number: CEB 10c

Principal Investigators: S. W. Rosen, Ph.D., M.D., B. D. Weintraub, M.D.

Other Investigators: A. S. Rabson, M.D., A. Tashjian, Jr., M.D., H. Sussman, M.D., F. Muggia, M.D., H. Hansen, M.D., I. Stotler, B.S., G. Krauth, M.S., Y. Kadesky, B.A., L. Panton, M.D., S. Krauss, Ph.D., E. Maxwell, Ph.D.

Cooperating Units: Pathologic Anatomy Dept., Clinical Center; Harvard School of Dental Medicine, Boston, Mass.; Pathology Dept., Stanford University Medical Center, Palo Alto, CA; NCI Oncology Unit, V. A. Hospital, Washington, D.C., Laboratory of Molecular Biology, NIAMDD

Man Years:

Total: 4 1/4
Professional: 2
Other: 2 1/4

Project Description:

I. Placental Protein "Markers"

A) In Vivo Studies

Serum and tumor extracts from a 75-year-old man with mixed epidermoid-adenocarcinoma of the pancreas contained the beta subunit of hCG (hCG-β) identified in three different radioimmunoassays and by gel chromatography. There was no evidence for either complete hCG or an alpha subunit, a situation somewhat analogous to isolated production of an immunoglobulin chain by certain lymphoid-plasma cell neoplasms. The questions of possible hetergeneity of this ectopic hCG-β is being studied.

Serum from a 27-year-old woman with metastatic gastric carcinoid had 20,000 ng/ml of an alpha subunit identified by radioimmunoassay and by gel chromatography; this material was also present in tumor extracts. Again, there was no evidence for any other subunit nor for remotely comparable amounts of a complete glycoprotein trophic hormone.

Both the alpha subunit and complete biologically active hCG were detected in the serum of still another cancer patient (47-year-old man with bronchogenic carcinoma), and tumor tissue from this patient has been successfully cultured in vitro (see below). If metabolic clearance rate data for the subunits in man is comparable to such data in rodents, in vivo tumor release of the alpha subunit exceeded its release of complete hCG, and is well reflected by the in vitro data for one of the clones (see below).

Prospective analysis of sera from 123 patients with bronchogenic carcinoma has revealed 7 new cases of ectopic placental protein production (hCG-4, hPL-1, PAP-1, hCG-β-1). The production of these placental proteins was discordant. Thus, the development of assays for additional placental proteins may prove useful in detection of certain cancers as well as in monitoring their response to therapy.

B. In Vitro Studies

We have recently studied a case of bronchogenic carcinoma associated with ectopic production of biologically active hCG in vivo. A metastatic tumor nodule removed from this patient incorporated radioactive amino acids into hCG in vitro as demonstrated by short-term explant studies. The same tumor was used to establish uncloned cell lines in tissue cultures (ChaGo lines), which were shown to secrete biologically active hCG into the medium. These uncloned cell lines have been maintained in serial culture for over three years and have continued to produce hCG. Recently, it has been demonstrated that these cells also secrete hCG-β as well as relatively large amounts of hCG-α.

In order to better define the origin of hCG and its subunits from these cells, we have established three clonal strains, which have been maintained in serial culture for over two years. Although each of the three clones secretes complete hCG, each secretes much larger amounts of either hCG-α or hCG-β, and the ratio of the two subunits produced varies greatly among the clones. Currently, studies are in progress to determine the mechanismm of unbalanced subunit production in these cells.

C. Purification of Polysomes Synthesizing Placental Proteins

We have prepared polysomes from term placenta as well as from in vitro lines of a human lung cancer which produces the free alpha subunit of chorionic gonadotropin. Free and membrane-bound polysomes were isolated from tissue homogenates by differential centrifugation in a sucrose gradient. Radioimmunoassy of the polysome preparations revealed large amounts of placental proteins primarily associated with the membranes bound to the polysomes. We are currently attempting to characterize the nascent proteins on fractionated polysomes in order to determine whether the messages for the alpha and beta subunits of chorionic gonadotropin are independently transcribed and translated. The function of the polysomes is also being studied by characterizing the labeled proteins synthesized in vitro after a pulse of labeled amino acids. We eventually plant to use these polysomes to isolate messenger RNA specific for various placent proteins.

Ectopic alpha subunits in tissue culture medium from bronchogenic carcinoma cell lines (ChaGo-α, see I-B above) and in a gastric carcinoid tumor (AL-α, see I-A above) were purified by ethanol-salt percolation on celite, gel and ion exchange chromatography. ChaGo-α partially purified from both an uncloned cell line and a single clonal strain displayed heterogeneity in polyacrylamide gel electrophoresis (PAGE) and gel chromatography, with a dominant component of higher apparent molecular weight (app MW) than hCG-α. Highly purified (>11,000-fold) AL-α was also heterogeneous in PAGE but was relatively homogeneous in gel chromatography and in sodium dodecyl sulfate-PAGE where its app MW (14,700 ± 400), was significantly less than hCG-α (23,800 ± 800), hLH-α, hFSH-α, or hTSH-α. The amino acid composition of AL-α was significantly different from identically analyzed hCG-α but there was a similar hexosamine content. In contrast to hCG-α, AL-α displayed only a minimal (1.4%) and delayed combination with hCG-β to form hCG active in a rat testis radioreceptor assay.

These studies indicate heterogeneity of purified ectopic alpha subunits derived from a single tumor in vivo or a single clonal strain in vitro. The principal components of ectopic alpha differ from normal alpha subunits in physical, chemical and hCG-β combination properties.

II. Apparent Cooperativity in Radioimmunoassay

We have found that selected antisera to hormones display enhanced binding of labeled hormone in the presence of small amounts of unlabeled hormone. Various binding analyses have shown that this phenomenon is analogous to other known cooperative protein-ligand interactions. This is supported by studies of antibody fragemtns but loss of this effect in enhanced binding of divalent $F(ab)_2$ fragements but loss of this effect in univalent Fab fragments. Studies are in progress to further define this phenomenon and to further explore its usefullness in radioimmunoassay.

Honors and Awards: None

Publications:

1. Rosen, S. W. and Weintraub, B. D.: Gonadotropins and Placental Proteins Produced by Human Tumors. Proceedings of the Fourth International Congress on Endocrinology, Washington, June 1972, Excerpta Medica, Amsterdam, pp. 1211-1215.

2. Weintraub, B. D. and Rosen, S. W.: Competitive Radioassays and "Specific" Tumor Markers. Metabolism 22: 1119-1127, 1973.

3. Weintraub, B. D. and Rosen, S. W.: Ectopic Production of the Isolated Beta Subunit of Human Chorionic Gonadotropin. J. Clin. Invest. 52: 3;35-3142, 1973.

4. Sussman, H. H., Weintraub, B. D. and Rosen, S. W.: Relationship of Ectopic Alkaline Phosphate to Ectopic Chorionic Gonadotropin and Placental

Lactogen: Discordance of Three "Markers" for Cancer. Cancer 33: 820-823, 1974.

5. Golde, D. W., Schambelan, M., Weintraub, B. D. and Rosen, S. W.: Gonadotropin-secreting Renal Carcinoma. Cancer 33: 1048-1053, 1974.

Serial No. NIAMDD/CEB 10c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Macro LDHemia

Previous Serial Number: CEB 11c

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigators: C. R. Kahn, M.D., R. Temple, M.D., D. Thomas, M.D., N. Papadopoulos, M.D., E. Vesell, M.D.

Cooperating Units: Clinical Pathology Dept., Clinical Center; Walter Reed Army Institute of Research; Dept. of Pharmacology, Hershey Medical Center, Hershey, PA

Man Years:

Total: 0
Professional: 0
Other: 0

Project Description:

Inactive

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 11c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies with Luteinizing Hormone-Releasing Factor (LRF)

Previous Serial Number: CEB 12c

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigators: A. D. Rogol, M.D., J. Rochefort, Ph.D.

Cooperating Units: Ayerst Laboratories, New York, New York

Man Years:

Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Synthetic decapeptide LRF (Ayerst) was administered to 12 patients (5F, 7M) with the syndrome of congenital anosmia and hypogonadism (the Kallmann Syndrome). Two men showed trivial release (∿720 MIU x min) for both LH and FSH. The majority (4M, 5F) had release of 1100-8000 for LH and 600-2300 for FSH. One man with spermatogenesis and erythrocyte phenotype-compatible paternity, displayed enhanced LH release (24,000) but FSH release was not different from the majority of responders.

The data are compatible with heterogeneity of response to LRF in this syndrome.

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 12c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Growth Hormone Studies

Previous Serial Number: None

Principal Investigator: S. W. Rosen, Ph.D., M.D.

Other Investigator: R. C. Dimond, M.D.

Cooperating Units: Walter Reed Army Institute of Research

Man Years:

Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Gel chromatography (Sephadex G-100) of serum from patients with acromegaly demonstrated three immunoactive components of circulating growth hormone (hGH). Peak II ($K_{av} = 0.3$) and Peak III ($K_{av} \simeq 0.4$) material eluted in positions similar to those described for "little" and "big" hGH, respectively. Peak I material eluted in the void volume and comprised ~20-35% of the total immunoactive hGH. On rechromatography, recovery was virtually quantitative and Peak I again co-eluted with an internal marker of the void volume (^{125}I-bTG).

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 13c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1973 through June 30, 1974

Project Title: TSH and Prolactin Studies

Previous Serial Number: 14c

Principal Investigators: B. D. Weintraub, M.D. (A-E), S. W. Rosen, Ph.D., M.D. (F-G)

Other Investigators: I. Kourides, M.D., F. Maloof, M.D., A. Tashjian, Jr., M.D. (A-E), A. Rogol, M.D., R. Dimond, M.D. (F-G)

Cooperating Units: Walter Reed Army Institute of Research; Harvard Medical School, Boston, Massachusetts

Man Years:

Total: 1 1/2
Professional: 1
Other: 1/2

Project Description:

A) Response of Prolactin and TSH to Suckling

New studies from our laboratory support previous data that TRH is probably not the physiologic mediator of the suckling response in postpartum women. A variety of natural and mechanical suckling stimuli consistently produce a rise in serum prolactin but not in serum TSH. Further studies revealed that there were no significant increases in free or total T_4 or T_3 in these women which would cause a selective blunting of the TSH response to TRH. Moreover, a combination of TRH and suckling similarly did not blunt the TSH response.

B) Measurement of TSH Subunits in Human Serum and Pituitary Glands

We have developed specific radioimmunoassays for both the alpha and beta subunit of human TSH and are currently studying the regulation of subunit production as reflected in pituitary content and serum concentration. Recently we have examined the relative concentrations of TSH, TSH-beta and the common alpha subunit in sera from hypothyroid patients before and after various doses of exogenous T_4 and T_3. These studies have revealed discordant dose responses between the two subunits and incomplete suppression of the alpha subunit. Measurement of free subunits in human pituitary gland extracts have revealed a large pool of alpha subunit relative to TSH-beta, which may indicate that the latter is limiting in TSH biosynthesis.

C) Metabolic Clearance and Production Rates of TSH

We have measured the metabolic clearance and production rates of human thyrotropin in a variety of patients with pituitary and thyroid disorders. The clearance rate of TSH is directly proportional to the serum concentration of T_3 and T_4 and therefore contributes to alteration in serum TSH. However changes in clearance rate occur later than changes in pituitary production rate.

D) Target Organ Resistance to Thyroid Hormone

We have discovered three new cases, since our original report, of partial target organ resistance to T_3 and T_4. Studies continue concerning the mechanism of the syndrome and its implications for the action of thyroid hormones. Currently we are attempting to define the long-term effects of pharmacologic doses of T_3.

E) Suppression of TSH Secretion by the Hyperfunctioning Thyroid Nodule

Solitary hyperfunctioning nodules are associated with TSH suppression, both basally and after administration of TRH, even in patients who are clinically and chemically euthyroid. The proposed mechanism for this phenomenon is secretion of amounts of T_4 and/or T_3 by the nodule which are slightly in excess of the normal for that individual patient. In support of this theory is the fact that after removal of such a nodule, thyroid hormone levels fall and TSH levels rise until a new state of TSH responsiveness is reached. This new steady-state is achieved in an individual patient with only a subtle fall in T_3 and T_4 levels which are well within the "normal" range of a large group of patients.

F) Radioiodination of Prolactin

We are currently attempting to further characterize the various radioiodination products of both bovine and human prolactin after chloramine T and lactoperoxidase procedures. It appears that various products have different molecular weight, charge, and immunoreactivity which may affect use of tracer in physicochemical studies and radioimmunoassay.

G) Heterogeneity of Circulating Human Prolactin

Multiple immunoactive components of circulating human prolactin (hPRL) were detected in 6 plasma samples analyzed by Sephadex G-100 chromatography. Prolactin of apparently large mol. wt. ($K_{AV} \simeq 0.2$) was the major (70%) circulating immunoactive prolactin in a patient with a pituitary tumor and acromegaly. Much less (< 10-20%) K_{AV} 0.2 prolactin was detected in the other plasmas drawn from volunteers before and after TRF, during pregnancy and postpartum lactation and from a patient with a chromophobe adenoma and galactorrhea. In these 5 plasmas, the major (80-90%) immunoactive component ($K_{AV} = 0.4$) was not distinguishable chromatographically from monomeric pituitary prolactin.

Honors and Awards: None

1. Gautvik, K. M., Weintraub, B. D., Graefer, C. T., Maloof, F., Zuckerman, J. E. and Tashjian, A. H., Jr.: Serum prolactin and TSH: effects of nursing and Pyro Glu-His-Pro NH_2 administration in postpartum women. J. Clin. Endocrinol. Metab. 37: 135-139, 1973.

2. Ridgway, E. C., Weintraub, B. D., Cevallos, J. L., Rack, M. C., and Maloof, F.: Suppression of pituitary TSH secretion in the patient with a hyperfunctioning thyroid nodule. J. Clin. Invest. 52: 2783-2792, 1973.

3. Ridgway, E. C., Weintraub, B. D. and Maloof, F.: Metabolic clearance and production rates of human thyrotropin. J. Clin. Invest. 53: 895-903, 1974.

4. Kourides, I. A., Weintraub, B. D., Ridgway, E. C. and Maloof, F.: Increase in the beta subunit of human TSH in hypothyroid serum after thyrotropin releasing hormone. J. Clin. Endocrinol. Metab. 37: 836-840, 1973.

5. Kourides, I. A., Weintraub, B. D., Levko, M. A. and Maloof, F.: Alpha and beta subunits of human thyrotropin: purification and development of specific radioimmunoassays. Endocrinology 94: 1411-1421, 1974.

6. Rogol, A. D. and Rosen, S. W.: Prolactin of apparent large molecular size: the major immunoactive prolactin component in plasma of a patient with a pituitary tumor. J. Clin. Endocrinol. Metab. 38: 714-717, 1974.

Serial No. NIAMDD/CEB 14c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1973 through June 30, 1974

Project Title: Cyclic Nucleotide-dependent Protein Kinases of the Thyroid Gland

Principal Investigators: M. McClung, M.D., J. E. Rall, M.D., Ph.D.

Other Investigators: None

Cooperating Units: None

Man Years:

Total: 1 3/4
Professional: 1 1/2
Other: 1/4

Project Description:

The mechanism by which thyroid stimulating hormone (TSH) stimulates a variety of responses in the thyroid gland has been shown to be mediated by cAMP. The sequence of events between the increased levels of cAMP and the altered cellular functions has not been elucidated, but very likely this involves cAMP-dependent protein kinases. Our project involves the identification and characterization of the cyclic nucleotide-dependent protein kinases of the thyroid gland.

Cytoplasmic protein kinase has been purified from bovine thyroid glands and has been characterized in this laboratory. It has been found to resemble cyclic nucleotide protein kinases from other tissues both in its cAMP-binding and its enzymatic activities. In addition, kinase activity was demonstrated in the particulate fraction of bovine thyroid homogenates. This activity has been further characterized in plasma membrane-enriched preparations. Membrane-associated protein kinase phosphorylates both exogenous substrates such as histone and protamine as well as endogenous substrates demonstrates cAMP-dependence while endogenous substrate phosphorylation does not. The enzymatic and cAMP-binding activities of the membrane-associated kinase are being further characterized.

Honors and Awards: None

Publications: None

Serial No. NIAMDD/CEB 15c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Reports
July 1, 1973 through June 30, 1974

Project Title: Antimitotic Agents and Adrenal Secretion

Previous Serial Number: CEB 22c

Principal Investigators: J. Wolff, M.D., Ph.D., R. Temple, M.D.

Other Investigators: G. H. Cook

Cooperating Units: None

Man Years:

Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Steroid secretion was studied in adrenal cells lacking a functional ACTH receptor. This line responds to antimitotic agents in a manner similar to that reported for the Y-1 line. Other stimuli were also examined. Choleragen, the pure protein from cholera toxin, stimulates steroid secretion by Y-1 adrenal tumor cells in culture. The secreted steroids are the same as seen after addition of adrenocorticotropic hormone. Half-maximal stimulation occurs at 15 pM; stimulation is essentially irreversible by washing and partially reversible (for about 1 hr) by antibody, and there is a latent period of about 60 min before stimulation is seen. Stimulation of adenylate cyclase occurs at about 30-fold higher choleragen concentrations. Gangliosides inhibit choleragen stimulation when added before but not after the toxin. Lipopolysaccharides from Escherichia coli, Salmonella typhosa, and Serratia marcescens also stimulate steroid secretion, but are less potent than choleragen. The defective line also responded to choleragen.

Honors and Awards: None

Publications:

1. Wolff, J., Temple, R. and Cook, G. H.: Stimulation of Steroid SEcretion in Adrenal Tumor Cells by Choleragen. Proc. Nat. Acad. Sci. USA 70: 2741-2744, 1973.

Serial No. NIAMDD/CEB 16c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
July 1, 1973 through June 30, 1974

Project Title: Thyroid Plasma Membranes

Previous Serial Number: 23c

Principal Investigators: J. Wolff, M.D., Ph.D., W. V. Moore, M.D., Ph.D.

Other Investigators: G. H. Cook

Cooperating Units: None

Man Years:

Total: 2 1/2
Professional: 1 1/2
Other: 1

Project Description:

In continuation of studies on beef thyroid membrane binding of [^{3}H]TSH and [^{125}I]TSH to beef thyroid membranes was studied under conditions to give optimum binding. Binding was maximal at pH 5.5, was complete within 5 min and was stable for at least 90 min at 0° and 22°C for low and high TSH concentrations, respectively. At pH 7.5, two binding sites were found with apparent affinity constants of 2.2-3.1 x 10^{8} M^{-1} and 0.7-1.1 x 10^{6} M^{-1}. Because of lower specific activity, [^{3}H]TSH was used primarily in the concentration range of the low affinity constant while [^{125}I]TSH was used at concentrations corresponding to both constants. Binding of [^{3}H]TSH and [^{125}I]TSH was equivalent when measured in the same concentration range.

TSH binding was inhibited by divalent cations (Ca^{2+}, Mg^{2+}, Sr^{2+}, Mn^{2+}, Ba^{2+}) at concentrations above 0.1 mM while monovalent cations inhibited binding at concentrations above 10-15 mM. The order of potency for inhibition of binding by monovalent cations was $Na^{+} \simeq K^{+} > Li^{+}$. Inhibition of binding by cations was independent of TSH concentration.

When binding was determined under adenylate cyclase assay conditions, TSH binding was reduced to approximately one-third of the control levels and the higher apparent affinity constant was reduced to 1.6 x 10^{7} M^{-1} whereas the second constant was unchanged. These constants coincide roughly with the midpoints of the concentration curve of TSH activation of adenylate cyclase.

TSH was not displaced from the membranes by ITP and GTP at concentrations which enhance cyclase but only at concentrations above 1 mM; the effect of

ITP and GTP was independent of medium conditions, TSH concentration or the presence of a regenerating system.

TSH binding studied in the presence of activators and inhibitors of adenylate cyclase permitted classification of the compounds into four groups: (1) agents which affect cyclase and TSH binding in a parallel fashion (Ca^{2+}, Na^{+}, Li^{+}, pronase, cobramine B); (2) agents which enhance cyclase but decrease TSH binding (K^{+}, Mg^{2+}); (3) agents which inhibit cyclase but enhance binding (phospholipase A, filipin); (4) agents which alter cyclase but not binding (ITP, GTP, F^{-}, chlorpromazine, gramicidin S, valinomycin, cetyl pyridinium chloride).

It is concluded that: (1) TSH binding is relatively stable and requires major structural alterations to affect binding; (2) the effect of activators and inhibitors on TSH binding is independent of TSH concentration and medium conditions; (3) most modulators act in the cyclase system at a stage beyond hormone binding; (4) the response of TSH binding to nucleotide triphosphates indicates that the thyroid membrane system is different from other membrane systems; and (5) the relationship between hormone binding and cyclase activation is complex and direct correlation between binding and activation is not presently possible.

Honors and Awards: None

Publications:

1. Moore, W. V. and Wolff, J.: Binding of prostaglandin E_1 to beef thyroid membranes. J. Biol. Chem. 248: 5705-5711, 1973.

Serial No. NIAMDD/CEB 17c
1. Clinical Endocrinology Branch
2. Endocrine Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Thyroid Hormone Secretion

Previous Serial Number: CEB 23c

Principal Investigators: J. Wolff, M.D., Ph.D., B. Bhattacharyya, Ph.D.

Other Investgators: None

Cooperating Units: None

Man Years:

Total: 1 1/2
Professional: 1 1/4
Other: 1/4

Project Description:

In order to understand the role of aggregation of tubulin subunits in the thyroid secretory process, the colchicine-binding protein was purified from beef thyroid glands with a procedure which allows isolation of this protein from a source low in tubulin. The protein dimer has a molecular weight of 114,000. Sodium dodecyl sulphate-acrylamide gel electrophoresis indicates that the subunits have molecular weights of 55,000 $\pm$ 2,000. Two monomeric subunits were identified by urea-acrylamide gel electrophoresis. Amino acid analysis show differences from other tubulins in lysine, aspartic and glutamic acid contents. The protein binds $\sim$0.85 moles of colchicine and contains 0.8 moles of alkali labile phosphate per mole of dimer. Combination of colchicine with the tubulin dimer induces marked fluorescence at 430 nm. This constitutes a convenient method for studying colchicine binding. On the basis of molecular weight, subunits, sedimentation constant, amino acid composition and colchicine-binding activity, the protein is very similar to the subunit protein of cilia, flagella and brain tubules.

In addition, it has been found that colchicine fluoresces upon binding to tubulin and that this interaction provides a convenient assay procedure for the protein.

Honors and Awards: None

Publications:

1. Wolff, J. and Williams, J. A.: Microtubules and Thyroid Secretion. Proceedings of the 4th International Congress of Endocrinology, 1972. Int. Congress Series No. 273: 294-298, 1973.

2. Wolff, J. and Williams, J. A.: The Role of Microtubules and Microfilaments in Thyroid Secretion. Rec. Prof. Horm. Res. 29: 229-285, 1973.

3. Bhattacharyya, B. and Wolff, J.: Thyroid Tubulin, Purification and Properties. Biochemistry 13: May, 1974.

Serial No. NIAMDD/CEB 18C
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Factors Affecting Insulin Receptor Concentrations

Previous Serial Number:

Principal Investigators: A. Soll MD.,C. R. Kahn MD, J. Roth MD

Other Investigators: D. M. Neville, Jr.

Cooperating Units: Laboratory of Neurochemistry, NIMH

Man Years:

Total: 1 1/2
Professional: 1
Other: ½

Project Description:

Studies have been conducted in a variety of animal models to determine the role of the insulin receptor in states of altered hormone sensitivity and the various factors affecting receptor concentration. In both liver membranes and thymic lymphocytes of the obese hyperglycemic mouse (ob/ob) we have demonstrated a decreased concentration of otherwise normal insulin receptors. In the thymic lymphocytes, there was a marked reduction in the effect of insulin on stimulation of α-aminoisobutyric acid influx which correlated with the marked decrease in receptors. The decrease in receptors was not unique to this model of obesity; it was also observed in the genetically obese db/db mouse and in the acquired obesity following gold thioglucose administration. In acquired obesity the decrease paralleled the increase in body weight and plasma insulin levels. Diet restriction produced an increase in receptors toward normal. However, if animals were maintained hyperinsulinemic during the diet, there was no improvement in the defect. Thus, chronic hyperinsulinemia appeared to be a major factor in causing a reduction in the concentration of insulin receptors.

Insulin receptor binding is also decreased in the insulin resistance of glucocorticoid excess whether induced by administration of steroids or by exogenous or endogenous ACTH. In contrast, growth hormone excess alone does not appear to effect receptor concentration. Hypophysectomy and adrenalectomy have minor effects to increase receptors.

Honors and Awards: None

Publications:

1. Goldfine, I. D., Kahn, C. R., Neville, D. M., Jr., Roth, J., Garrison, M. M., and Bates, R. W.: Decreased binding of insulin to its receptors in rats with hormone induced insulin resistance. Biochem. Biophys. Res. Commun. 53: 852-857, 1973.

2. Neville, D. M., Jr., Kahn, C. R., Soll, A., and Roth, J.: Plasma membrane insulin receptor regulation by genetic, metabolic and hormonal factors. In Peters, H. (ed.): Protides of the Biological Fluids, 21st Colloquium. New York, Pergamon Press, 1973, pp. 269-273.

Serial No. NIAMDD/CEB 19C
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: The Interaction of Insulin with Receptors in Liver

Previous Serial Number: 15c

Principal Investigators: C. R. Kahn, M.D., A. Soll, M.D., J. Roth, M.D.

Other Investigators: D. M. Neville, Jr., M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH

People Years:

Total: 1½
Professional: 1
Other: 1/2

Project Description:

The quantitative aspects of the insulin receptor interaction have been studied with normal rat and mouse liver membranes and with liver membranes from the genetically obese (ob/ob) mouse. Both equilibrium and kinetic studies suggest that the interaction is complex. The kinetics of association and dissociation are dependent on temperature, ionic strength of buffer, and concentration of the reactants. In addition, the rate of dissociation of ^{125}I-insulin from liver membranes is influenced by the presence of unlabeled insulin in the incubation media suggesting site-site interactions of the type called negative cooperativity. Scatchard analysis of the equilibrium binding data results in a curvilinear plot concave upward, while the Hill plot is linear with a slope of 0.8. Both of these are consistent with either heterogeneity of receptor sites or negative cooperativity.

In addition to the binding reaction, the liver membranes have been shown to possess both insulin and receptor degrading systems. The insulin degrading system has been previously characterized. Receptor degradation, like the binding reaction, is dependent on time, temperature, ionic strength of buffer and membrane concentration.

Comparison of insulin receptors from normal and obese mice suggests no significant differences in any of these parameters, with the exception of a decreased number of receptors in the obese mouse membranes.

Honors and Awards: None

Publications:

1. Neville, D. M., Jr. and Kahn, C. R.: Isolation of plasma membranes for cell surface membrane receptor studies. In Laskin, A. I. and Last, J. A. (eds.): Methods in Molecular Biology, Vol. 5: Subcellular Particles, Structures, and Organelles. New York, Marcel Dekker, 1974, pp. 57-88.

2. Kahn, C. R., Freychet, P., Roth, J. R., and Neville, D. M., Jr.: Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J. Biol. Chem. 249: 2249-2257, 1974.

Serial No. NIAMDD/CEB 20C
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of Nonsuppressible Insulin-like Activity (NSILA-s) of Plasma

Previous Serial Number: none

Principal Investigators: D. M. Neville Jr, M.D., C. R. Kahn, M.D.
J. Roth, M. C.

Other Investigators: D. M. Neville Jr., M.D., E. R. Froesch, M.D.
R. E. Humbel, M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH; Department of Medicine and Institute of Biochemistry, University of Zurich, Switzerland

People Years:

Total: 1 1/2
Professional: 1 1/2
Other: 0

Project Description:

NSILA-s is a partially purified serum peptide with insulin-like bioactivity but not immunoreactivity. NSILA-s reacts with insulin receptors in both liver and lymphocytes in direct proportion to its insulin-like potency. It is also a potent inhibitor of the degradation of ^{125}I-insulin by the insulin degrading proteases of hepatic plasma membranes. Using ^{125}I-NSILA-s, we have demonstrated another set of rceptors in liver that bind NSILA-s. These receptors have a high affinity for NSILA-s and a much lower affinity for insulin. NSILA-s recovered from these receptors may be purified by 2-3 fold.

NSILA-s in plasma was measured using this radioreceptor assay after gel filtration of plasma on Sephadex G-50 in 1 M acetic acid. In normal adults, plasma NSILA-s was 1200-2200 ng/ml. Elevated NSILA-s concentrations were detected in 5 of 7 patients with hypoglycemia in association with non-islet cell tumors. Plasma NSILA-s was not elevated in other patients with similar tumors but without hypoglycemia or in insulin-induced hypoglycemia. This suggests a possible role for NSILA-s in the etiology of the hypoglycemia associated with extrapancreatic tumors.

Honors and Awards: None

Publications:

1. Megyesi, K., Kahn, C. R., Roth, J., Froesch, E. R., Humbel, R. E., Zapf, J. and Neville, D. M., Jr.: Insulin and non-suppressible insulin-like activity (NSILA-s): Evidence for separate plasma membrane receptor sites. Biochem. Biophys. Res. Commun. 57: 307-315, 1974.

2. Megyesi, K., Kahn, C. R., Roth, J. and Gorden, P.: Hypoglycemia in association with extrapancreatic tumors: Demonstration of elevated plasma NSILA-s by a new radioreceptor assay. J. Clin. Endo. 38: 931-934, 1974.

Serial No. NIAMDD/CEB 21c
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Effect of Insulin and Polypeptides with Insulin-like Activity on DNA Synthesis in Cultured Human Fibroblasts

Previous Serial Number: CEB 25c

Principal Investigator: M. M. Rechler, M.D.

Other Investigators: C. A. Wells, Ph.D. and J. Podskalny

Cooperating Units: P. Nissley, National Cancer Institute

People Years:

Total: 3
Professional: 2
Other: 1

Project Description:

Non-suppressible insulin-like activity (NSILA-s) is an acid-soluble polypeptide of molecular weight 7000 with insulin-like metabolic activity that is not neutralized by antibodies to insulin. Highly purified preparations of NSILA-s, like insulin stimulated [^{3}H]thymidine incorporation into DNA by cultured human fibroblasts. NSILA-s was effective at concentrations that have been found in human serum, whereas insulin was active only at high concentrations. Combined addition of maximally effective concentrations of insulin and NSILA-s failed to give additive stimulation, suggesting that the two polypeptides act by a common mechanism. In contrast, combined addition of insulin and serum, or NSILA-s and serum, gave additive responses.

In conjunction with P. Nissley (NCI), we have studied a polypeptide fraction designated multiplication-stimulating activity (MSA) that is present in medium conditioned by certain cultures of Buffalo rat liver cells. MSA is similar to NSILA-s with respect to molecular weight, acid solubility, insulin-like metabolic activity that is not neutralized by insulin antibodies, and the ability to stimulate cell growth and DNA synthesis. MSA stimulates [^{3}H]thymidine incorporation in chick embryo and human fibroblasts. MSA competes effectively with ^{125}I-NSILA-s for binding to NSILA-s receptors of rat liver membranes (performed by K. Megyesi), further suggesting a close relationship between MSA and NSILA-s. Like NSILA-s, MSA did not stimulate [^{3}H]thymidine incorporation by human fibroblasts, additively with insulin; stimulation by MSA and serum was additive. We have been unable to demonstrate any effect of growth hormone on MSA production in any of the cultured rat liver tested.

Dibutyryl cyclic AMP (Dbc) blocks the stimulation of human fibroblast DNA synthesis by serum or insulin. Maximal inhibition is observed when Dbc is added together with serum, or up to 7 hours later, suggesting that Dbc affects a relatively late step in DNA synthesis. Addition of Dbc together with serum, followed by replacement with fresh serum-containing medium, does not delay the wave of DNA synthesis. Dbc exerts selective effects on DNA synthesis, since $< 20\%$ inhibition of RNA synthesis is seen when DNA synthesis is inhibited $> 90\%$. Dbc does not affect [^{3}H]thymidine uptake from the medium or the phosphorylation of thymidine to dTTP.

Honors and Awards: None

Publications:

1. Rechler, M. M., Podskalny, J. M. and Wells, C. A.: DNA synthesis in human fibroblasts: Stimulation by insulin and by nonsuppressible insulin-like activity (NSILA-s). J. Clin. Endo. Metab., 1974, in press.

Serial No. NIAMDD/CEB 22C
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Cooperative Mechanisms Regulating Receptors' Affinity for Insulin

Previous Serial Number: None

Principle Investigators: Pierre de Meyts, M.D., Jesse Roth, M.D.

Other Investigators: David M. Neville, Jr., M.D., Maxine A. Lesniak

Cooperating Units: NIMH, Laboratory of Neurochemistry

People Years:

Total: 1½
Professional: 1
Other: 0½

Project Description:

We have designed an original method to detect cooperative interactions, based on differences in the dissociation rate constant studied by "infinite dilution" of the labeled hormone-receptor complex in the absence and in the presence of unlabeled hormone. Classical kinetics predicts that the dissociation rate of the tracer should be in this experimental situation independent of the presence of unlabeled hormone in the dilution medium. This proved to be the case for the growth hormone-receptor system in human cultured lymphocytes. In the case of the insulin receptor in lymphocytes the dissociation rate of the tracer is dramatically increased by the presence of unlabeled insulin; the halt-time for dissociation in the presence of unlabeled is one--tenth of the one by dilution only; this demonstrates the existence of site--site interactions upon filling of empty sites by the unlabeled hormone (negatively cooperative binding).

The application of this method to the study of various insulin analogues has led us to some important conclusions regarding the structural specificity of insulin bioactivity. We have studied for their ability to induce the site-site interactions 25 insulin analogues, representing altogether modifications of 65% of the primary structure. We have shown that most of the insulin analogues affect the cooperative interactions in direct proportion to their ability to occupy the receptors while two analogues, desalanine-desasparagine insulin and desoctapeptide insulin produced no cooperative interactions even at concentrations at which they saturate all the sites and one, despentapeptide insulin, showed only 15% of the expected effect. We thus propose

that insulin binding to receptor sites involved two discrete regions on both molecules: one region related to bioactivity in which binding occurs in direct proportion to the biological potency of the hormone analogue, and a second regulatory site which involves a discrete part of the insulin molecule in the region of the A_{21} asparagine and the B22-30 octapeptide. Binding of this regulatory site affects site-site interactions, decreasing the affinity of other sites for insulin.

We also have evidence that insulin dimers bind to the insulin receptor but do not exert negative cooperativity. Concanavallin A is a potent inhibitor of the cooperative effect induced by insulin binding, without being a competitive inhibitor of the binding itself.

Serial No. NIAMDD/CEB 23C
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Acromegaly and Relationship to Heart Disease and Visceromegaly

Previous Serial No.: None

Principal Investigator: William L. McGuffin, Jr., M.D., Jesse Roth, M.D. and Philip Gorden, M.D.
Other Investigators: C. Ronald Kahn, M.D., William Roberts, M.D.

Cooperating Units: NHLI, Peter Fromer, M.D.

People Years:

Total: 1.0

Professional: 1.0

Other: none

Project Description:

A prospective study of acromegaly has been in progress for 10 years. One of the areas studied was the relationship of acromegaly to heart disease. We found that cardiomegaly, while routine at necropsy, is not out of proportion to the increase in size of other internal organs and is not detected clinically except in patients with either hypertension or CHF. Hypertension was found in 23% of acromegalics and was usually mild, uncomplicated, and easily treated with standard anti-hypertensive drugs. Some form of cardiac abnormality was detected in 30% of acromegalics. In half of these the abnormality was an EKG abnormality in an asymptomatic patient. Nine patients (16%) had symptomatic heart disease including 4 with arrhythmias of whom 3 were hyperthyroid, 2 with coronary heart disease, 2 with a cardiomyopathy, and one with transient CHF. Seven of these nine had CHF. Except for the hypertension, there was no correlation between the presence or absence of heart disease and the hGH level. A reduction in hGH levels by an average of 80% did not influence the hypertension or cardiac disease except in one patient in whom the CHF became easier to manage. We concluded that an aggressive approach to the reduction in hGH levels is not usually indicated solely on the basis of co-existent cardiac disease.

A second area of study has been the relationship of acromegaly with enlargement of other internal organs. Clinical enlargement of the liver or spleen could not be attributed to acromegaly per se but reflected another disease process. Therefore, the presence of hepato- or splenomegaly in an

acromegalic warrants an evaluation just as it would in the nonacromegalic. Parotid gland enlargement, however, was commonly found and could be attributed only to the acromegaly.

Awards: None

Publications:

1. McGuffin, W. L., Jr., Sherman, B. M., Roth, J., Gorden, P., Kahn, C. R., Roberts, W. C. and Frommer, P. L.: Acromegaly and cardiovascular disorders: A prospective study. Ann. Int. Med. 81: 1974.

2. Sober, A. J., Gorden, P., Roth, J. and A , . W.: Visceromegaly in acromegaly: Evidence that clinical hepatomegaly or splenomegaly (but not sialomegaly) are manifestations of a second disease. Arch. Int. Med., 1974

Serial No. NIAMDD/CEB 24C
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Interaction of Polypeptide Hormones with their Receptors in Target Tissues

Previous Serial Number:

Principle Investigators: Maxine A. Lesniak, Jesse Roth, M.D., A. Raffaele Bianco, M.D., William L. McGuffin, M.D., Philip Gorden, M.D.

Other Investigators: Pierre De Meyts, M.D., Carla Hendricks, Juanita A. Archer, M.D., David M. Neville, Jr., M.D., Donald N. Buell, M.D., Ronald Schwartz, M.D.

Cooperating Units: NIMH, NCI, Howard University

People Years:

Total: 5
Professional: 1.0
Other: none

Project Description:

I. The interaction of human growth hormone with cultured human lymphocytes has been characterized in a sensitive radioreceptor assay. Human growth hormone preparations of varying potencies which were studied in immunoassay and bioassay were also studied in the radioreceptor assay. The potencies of these hGH preparations determined by radioreceptor assay correlated more closely with the bioassay than with the radioimmunoassay.

II. When human plasma is characterized on Sephadex G-100, 2 discrete immunoreactive peaks designated "big" and "little" GH are found. "Big" GH in both normal subjects and acromegalic patients have less than 20% radioreceptor (RRA) activity as compared to radioimmunoassay (RIA) activity. The "little" GH component in normal subjects has a RRA/RIA activity less than RRA/RIA for acromegalic patients.

III. Chronic exposure of cultured lymphocytes to 10^{-8} M insulin at 37° *in vitro* produced a decrease in insulin receptor sites per cell, acute exposure of cells to insulin had no effect. These results reproduced observations *in vivo* in obese mice which have 10^{-8} M circulating insulin and are insulin resistant. Receptor concentration seems to be dependent on the

insulin concentration in an inverse relationship, e.g. 10^{-8} M insulin reduces the receptor concentration, when insulin is removed from the medium the receptor concentration is restored to normal level in 24 hours. A metabolic inhibitor such as cycloheximide interferes with insulin receptor reappearance.

IV. Chronic exposure of cultured lymphocytes to 10^{-10} M-hGH at 37° produces a decrease in growth hormone receptor concentration. This is a reversible phenomenon, i.e. when GH is removed from the medium the receptor concentration returns to near normal concentration after 24 hours. The addition of a protein synthesis inhibitor interferes with reappearance of the GH hormone receptors but not with the viability of the cultured cells.

V. Specific growth hormone receptors have been characterized in cultured human lymphocytes. When these lymphocytes are incubated in phosphate-buffered saline ate cell concentrations of over 2 X 10^8 cells/ml at 30 for 90 minutes, the growth hormone receptors are released into the media. Over 80% of the receptors are lost in this incubation and about 40% are recovered in the supernatant. The receptors isolated in this manner retain the specificity observed in the intact cells, however, they appear to have lost some of their affinity. The hormone is only minimally degraded in the presence of the receptor solution. The hormone that has been complexed with the receptor appears to be purified as measured by its ability to rebind to intact cells. The receptor is at least in part a protein with a relatively high molecular weight (excluded on Sephadex G-200).

VI. We have shown the existence of insulin receptors on peripheral leukocytes. More specifically we have presently shown that 80% of the binding of the hormone by preparations of mixed mononuclear peripheral leukocytes are accounted for by the monocyte fraction. Applying techniques to enrich the monocyte fraction we obtained direct evidence by autoradiography that the binding of ^{125}I-insulin is to the monocytes.

Honors and Awards: None

Publications:

1. Gavin, J. R., III, Roth, J., Neville, D. M., Jr., De Meyts, P. and Buell, D. N.: Insulin-dependent regulation of insulin receptor concentrations: A direct demonstration in cell culture. Proc. Nat. Acad. Sci. U.S.A. 71: 84-88, 1974.

2. Lesniak, M. A., Gorden, P., Roth, J. and Gavin, J. R., III: Binding of ^{125}I-human growth hormone to specific receptors in human cultured lymphocytes. J. Biol. Chem. 249: 1661-1667, 1974.

3. Gorden, P., Lesniak, M. A., Hendricks, C. M. and Roth, J.: "Big" growth hormone components from human plasma: Decreased reactivity demonstrated by radioreceptor assay. Science 182: 829-831, 1973.

Serial No. NIAMDD/CEB 25C
1. Clinical Endocrinology Branch
2. Diabetes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Studies of Nonsuppressible Insulin-like Activity (NSILA-s) of Plasma

Previous Serial Number: none

Principle Investigators: K. Megyesi, M.D., C. R. Kahn, M.D., J. Roth, M.D.

Other Investigators: D. M. Neville, Jr., M.D., E. R. Froesch, M.D., R. E. Humbel, M.D.

Cooperating Units: Laboratory of Neurochemistry, NIMH; Department of Medicine and Institute of Biochemistry, University of Zurich, Switzerland

People Years:

Total: 1½
Professional: 1½
Other: 0

Project Description:

NSILA-s is a partially purified serum peptide with insulin-like bioactivity but not immunoreactivity. NSILA-s reacts with insulin receptors in both liver and lymphocytes in direct proportion to its insulin-like potency. It is also a potent inhibitor of the degradation of ^{125}I-insulin by the insulin degrading proteases of hepatic plasma membranes. Using ^{125}I--NSILA-s, we have demonstrated another set of receptors in liver that bind NSILA-s. These receptors have a high affinity for NSILA-s and a much lower affinity for insulin. NSILA-s recovered from these receptors may be purified by 2-3 fold.

NSILA-s in plasma was measured using this radioreceptor assay after gel filtration of plasma on Sephadex G-50 in 1 M acetic acid. In normal adults, plasma NSILA-s was 1200-2200 ng/ml. Elevated NSILA-s concentrations were detected in 5 of 7 patients with hypoglycemia in association with non-islet cell tumors. Plasma NSILA-s was not elevated in other patients with similar tumors but without hypoglycemia or in insulin-induced hypoglycemia. This suggests a possible role for NSILA-s in the etiology of the hypoglycemia associated with extrapancreatic tumors.

Honors and Awards: None

Cystic fibrosis of the pancreas (CF) and Respiratory Distress Syndrome (RDS) have been the objects of investigation by the PMB in 1973.

A. CYSTIC FIBROSIS OF THE PANCREAS (CF)

I. Biochemical and Metabolic Studies

a) Tissue culture studies

The methylation of transfer and ribosomal ribonucleic acid in human fibroblasts: Normal methylation of ribonucleic acid in cystic fibrosis: It has been reported that tissue-cultured fibroblasts and lymphocytes from patients with CF as well as obligatory heterozygotes for CF show a decreased methylation of RNA. If undermethylation of CF cells could be consistently demonstrated, a valuable and easily measurable marker for the detection of CF carriers would be available.

Therefore, a comprehensive study was undertaken of tRNA and 18S and 28S rRNA methylation in human fibroblasts. Cells from normal volunteers and patients with cystic fibrosis were examined during exponential growth by incubation with L-(methyl-^{14}C) methionine and (^{3}H) uridine. The ratio of ^{14}C to ^{3}H counts per minute in isolated RNA serves as a measure of the extent of methylation and was found to be similar in normal and CF fibroblasts for tRNA, and 28 S rRNA. Hydrolyzed samples of (^{14}C)-methyl-labeled RNA species were analyzed by two dimensional chromatography and autoradiography. The methylated base and nucleoside compositions of tRNA and rRNA agree well with data for HeLa cells and other eukaryote cells such as those from yeast, mouse, and chicken. RNA methylation patterns in CF fibroblasts were qualitatively and quantitatively similar to those observed in normal cells for 4S, 18S, and 28S RNA. There is no evidence that RNA species from patients with cystic fibrosis are deficient in any methylated components. Methylation of RNA by tissue-cultured cells from cystic fibrosis patients is qualitatively and quantitatively normal and will not prove to be useful as a marker for identification of carriers of cystic fibrosis. (Dr. P. Farrell and Dr. M. Klagsbrun, NIAMDD).

b) Genetic markers in cystic fibrosis

Alteration in blood polyamines in cystic fibrosis patients and CF heterozygotes: A recent report by Rennert *et al.* suggests that blood polyamine levels may be altered in cystic fibrosis. Specifically these investigators observed in a limited number of samples that spermidine was elevated twofold in blood from CF patients and heterozygotes. If such an alteration were consistently demonstrable in the latter group, measurement of polyamines might serve as a CF carrier detection test. Accordingly, we have undertaken an evaluation of polyamine metabolism in CF by initially measuring blood spermine (Sp) and its precursor, spermidine (Spd) in CF patients and obligate heterozygotes (CF-Hz).

For this purpose, using plastic containers wherever possible to maximize recovery, citrated whole blood was extracted twice with 3% perchloric acid. Subsequently, polyamines were concentrated by lyophilization, separated by ion exchange chromatography, and quantitated with ninhydrin using an amino acid analyzer. Utilizing these techniques, consistent recoveries (68 ± 1%, mean ± SEM, N = 6) of spermidine and spermine were obtained as determined by addition of ^{14}C-labeled compounds.

Group	No.	Spermidine	Spermine	Spd/Sp
Control A	10	6.89 ± .49	6.82 ± .73	1.06 ± .07
Control B	3	6.32 ± .91	3.25 ± .35	1.93 ± .06
CF Heterozygote	10	7.72 ± .61	4.71 ± .41	1.73 ± .16
Cystic Fibrosis	10	7.42 ± .44	5.22 ± .49	1.48 ± .08

The data, given as nmoles/ml whole blood (mean ± SEM), indicated that control samples were divisible into two groups, the majority of which (Control A) showed approximately equal concentrations of Spd and Sp. The second group of three samples (Control B) exhibited a Spd/Sp ratio (1.93) above the 95% confidence limit of group A. CF homozygote and heterozygote blood generally showed increased Spd and decreased Sp leading to significantly altered Spd/Sp ratios ($p<.005$ in each case as compared with the mean of control group A). Eight of the ten CF-Hz ratios were beyond the 95% confidence interval of control group A and the CF-Hz samples as a whole were significantly different ($p<.05$) than the total group of 13 controls.

In conclusion, it appears that blood levels of polyamines are disturbed in cystic fibrosis homozygotes and heterozygotes. Further study will be required to determine if this apparent abnormality will be useful in population screening for carriers of the CF genotype. In addition, in the view of the widespread distribution of polyamines in cells and tissues as well as their probable abnormality in tissue culture fibroblasts, further investigations may provide an insight as to the nature of the elusive basic defect in cystic fibrosis. (Dr. D. Lundgren (guest worker) and Dr. P. Farrell, NIAMDD).

Biochemical assessment of ciliary activity in the presence of cystic fibrosis serum: Since Spock, *et al.* (Pediat. Res. 1:173, 1967) first observed that serum from CF patients and heterozygotes induced ciliary dysrhythmia, several "CF factor" bioassays have been described. Such methods, however, suffer from several disadvantages in that they are: a) subjective, b) qualitative, and c) difficult to reproduce. We ourselves had consistently negative experiences with both rabbit tracheal (RT) and oyster gill (OG) assays. In an effort to develop a quantitative chemical assay, we have tried a fresh approach by measuring motility-coupled ciliary ATP hydrolysis in the presence of serum. In addition, to characterize the ATPase activity several basic studies were performed with OG and RT cilia. Homogeneous, motile cilia were prepared from the former by mincing in 0.05 M Tris HCl (pH 8.0), 0.3 mM EDTA; this preparation, which on electron microscopy showed intact cilia, hydrolyzed ATP in proportion to calculated beat frequency. A high molecular wt. ATPase was purified from RT cilia and shown to have properties similar to flagellar dynein.

The effect of various factors (time, temp., pH, cations, and substrate concentration) on ciliary ATP hydrolysis was determined. Explants, homogenates, and minces of RT and OG were preincubated for 30-45 min with either normal, CF, or CF heterozygote sera; subsequent addition of ATP-γ-^{32}P and measurement of ^{32}Pi production revealed that the latter two groups could not be distinguished from normal serum. Studies are continuing but this objective, quantitative assay has thus far failed to demonstrate the "CF factor." (Dr. P. Farrell, G. Fox (guest worker), NIAMDD; Dr. S. Spicer, Medical Col. of S. Carolina, Charleston).

c) Vitamin E deficiency in man

Effects of vitamin E deficiency in man: Protean manifestations of tocopherol deficiency occur in animals but man has not been shown to develop clinical symptoms. Previous studies have been hampered by the small population of subjects available for investigation. Patients with cystic fibrosis provide the best available model of vitamin E deficiency in the human. In order to evaluate the effects of tocopherol deficiency in man, we have focused on some of the main disturbances found in animals as well as performing electron microscopic studies. *Plasma and α-tocopherol* (α-T): Plasma α-T, determined after thin layer chromatography, was uniformly decreased in 50 patients with pancreatic achylia due to CF (mean = 125 µg/100 ml, range = 23-410; control mean = 730, range = 550-960). Erythrocyte α-T was decreased in parallel fashion and averaged 42 µg/100 ml of packed red cells in 5 patients as compared with a normal mean of 230 ± 13 µg/100 ml (Bieri and Poukka, Int. J. Vit. Res. 40:344, 1970). Oral supplementation with 3-5 IU/kg/day of water miscible α-T resulted in normal plasma levels and in a limited number of cases at autopsy normal concentrations of α-T in muscle and various other organs. *Hemolysis in vitro* by an improved H_2O_2 technique invariably accompanied vitamin E deficiency and showed a sigmoidal relationship to plasma α-T level. Determination of ^{51}Cr-RBC survival revealed a mean $t_{\frac{1}{2}}$ of 22 days in 19 deficient patients, with 3 subjects particularly low at <18 days; the average $t_{\frac{1}{2}}$ in 6 CF patients after α-T supplementation increased from 19 to 27 days. *Muscle involvement:* Creatinuria was present in 13 of 25 patients studied, with the total group of 25 showing an average creatine excretion of 200 mg/24 hr (normal mean = 20 mg/24 hr, $p<.01$). Elevated plasma aldolase and CPK (the skeletal muscle isozyme) have been found in 2 out of 40 patients thus far. Histological, histochemical, and electron microscopic studies of skeletal muscle taken from CF vitamin E deficient patients at biopsy are presently underway, but although some abnormalities have been detected, it seems unlikely that vitamin E deficient humans are susceptible to the marked necrosis of striated muscle found in several laboratory animals.

Subcellular membranous structures in intestinal biopsy samples were examined in double-blind fashion and were similar in appearance for control subjects and tocopherol deficient patients. Thus, despite the apparent susceptibility of membranes to oxidation damage in vitamin E deficient animals, integrity of such structures appears to be maintained in humans at least insofar as we can judge with this technique.

In conclusion, these investigations which are still in course indicate so far that vitamin E deficiency in man is associated with many of the

findings present in animals, but generally at a subclinical level. Such findings are: 1) Hemolysis, both *in vitro* and *in vivo* which can be corrected with administration of vitamin E or the addition of tocopherol to the *in vitro* system. 2) Evidence of muscle involvement as shown by creatinuria, occasional elevation of plasma activities of muscle derived enzymes, and deposition of ceroid pigment in smooth muscle. 3) The electron microscopic studies conversely indicate no visible difference in subcellular organelles in E deficient patients as compared with controls. However, it is possible that membrane changes in cellular organelles might occur, but not be of sufficient magnitude to give rise to visible morphologic changes.

The metabolic role of this ubiquitous biological antioxidant is still unclear, but its widespread presence in human tissues must serve a purpose. Patients with malabsorption and secondary tocopherol deficiency should, therefore, receive supplements of vitamin E. Such supplementation should also be considered in the dietary makeup of populations in areas of the world (e.g., Bangladesh, Jordan, etc.) suffering from malnutrition and receiving inadequate diets. However, there is little reason to add vitamin E supplements in countries such as the United States where people ingest adequate amounts of the vitamin in their diets and have normal blood tocopherol levels. This should be kept in mind, especially in view of the current public fad over vitamin E and its interest in "megavitamin therapy." (Dr. P. Farrell, Dr. R. Wood, Dr. J. Bieri, Dr. P. di Sant'Agnese, NIAMDD: Dr. W. Engel, NINDB; Dr. V. Fischer, Univ. of St. Louis, Mo.).

II. Clinical Studies

a) Tracheal mucociliary transport in cystic fibrosis and its modification by a pharmacological agent: Chronic pulmonary disease, secondary to bronchiolar and bronchial obstruction and bacterial infection, is the major cause of morbidity and mortality in cystic fibrosis. It has long been assumed that this was due to increased viscosity of pulmonary secretions with subsequent decreased mucociliary airway clearance. Recently, *in vivo* studies of mucociliary airway clearance have suggested that clearance rates in CF patients were in fact faster than normal (New Eng. J. Med. 288:651, 1973).

We have studied tracheal mucus velocity in patients with cystic fibrosis by a new technique (J. Appl. Physiol. 34:495, 1973). A fiberoptic bronchoscope is passed into the trachea of conscious volunteers under topical lidocaine anesthesia. Teflon discs are then blown onto the tracheal mucosa and their movement filmed with a movie camera. The discs are 0.67 mm in diameter and weigh 0.13 mg, well within the transport capabilities of the mucociliary system. The rate of movement is calculated from the change in image size of each disc with time, and is accurate within 10%. Xylocaine has been shown not to alter mucus velocity in dogs and sheep.

The average rate of mucus velocity in 14 patients with CF (ages 18-43, mean = 25) was 2.6 ± 3.3 mm/min (std. dev.). This was markedly slower than the rate in 20 normal volunteers (ages 18-44, mean = 27): 20.1 mm/min ± 6.4 (std. dev.) ($p<.001$). There was no co-relation of mucus velocity with age, sex, clinical status or pulmonary functions.

Following the measurement of mucus velocity, each subject was given 0.25 mg Terbutaline, a new beta-adrenergic agent, by subcutaneous injection. Fifteen minutes later the mucus velocity was measured again. There was no significant effect in normal volunteers, but in the CF patients the mucus velocity increased to 5.5 mm/min ± 3.6 mm/min ($p<.001$).

This study is the first to measure by a direct method the rate of mucociliary airway clearance in patients with CF, and to demonstrate its impairment in this disease. Furthermore, it is the first to demonstrate pharmacological improvement of mucus clearance in CF. Both these observations have potential therapeutic as well as theoretical implications, and may lead to a greater understanding and better treatment of cystic fibrosis. (Dr. R. Wood, Dr. P. Farrell, NIAMDD; Dr. A. Wanner, Mt. Sinai Hos. Med. Cen., Miami Beach, Fla.).

b) Pseudomonas vaccine in cystic fibrosis: Colonization of the respiratory tract with Pseudomonas aeruginosa occurs in most patients with cystic fibrosis (80% of the patient population followed at the NIH). This contributes to the morbidity and mortality in CF by increasing bronchial obstruction and leading to bronchiectasis and necrotizing pneumonia. Pseudomonas organisms are notoriously resistant to most of the commonly used antibiotics, and therefore treatment may be difficult. Enhancement of the humoral and cellular pulmonary defense mechanisms of the patients may be another way of approaching the clinical problem of Pseudomonas infection in CF.

We have employed a multivalent lipopolysaccharide antigen (Pseudogen, Parke-Davis Co.) to study the antibody responses of 12 CF patients. Each patient was given 6 doses of the vaccine intramuscularly over a two-week period. Although side effects did occur (fever, malaise, local tenderness, and induration) no patient had to be withdrawn from the study because of adverse reactions to the vaccine. Serum hemagglutinin titers to each of the seven serotypes contained in the vaccine were determined before and at intervals following the immunization. Good antibody responses, i.e., significant antibody titer increases, were found in all patients. Elevated titers persisted for 3-6 months and then returned to baseline values.

In the small group of patients studied, no objective improvement in pulmonary function or clinical status was noted. Pseudomonas was not eradicated from any patient's sputum. Two patients had what they felt were significant, albeit subjective, improvements for several months following immunization.

We are currently investigating the use of the vaccine by an intranasal route, in the hope of stimulating production of a local antibody response in the respiratory tract. Antibody responses (IgG, IgM, and sIgA) are being evaluated in sputum, bronchial washings (in some patients), serum, and parotid saliva.

We conclude from our studies to date that although good antibody responses can be achieved without serious side effects, there is no significant clinical response in patients in whom Pseudomonas colonization is already well

established. Clinical trials may be warranted in a larger number of patients, concentrating on those in whom Pseudomonas colonization has not yet occurred. (The intranasal route for immunization would seem well suited for this approach, if it is effective in producing significant antibody responses.) (Dr. R. Wood, NIAMDD; Dr. H. Reynolds, Dr. J. Pennington, NIAID).

B. RESPIRATORY DISTRESS SYNDROME (RDS)

Lecithin synthesis in the fetal lung as related to respiratory distress syndrome: Considerable evidence has accumulated suggesting that atelectasis in RDS develops as a result of deficient pulmonary surfactant, particularly lecithin (Pediatrics 40:13, 1967). In our studies, biochemical regulation of lecithin synthesis has been evaluated in fetal lungs from various species. Recently it has been found that by obtaining samples of amniotic fluid of mothers during gestation and assessing the content of lecithin which rises rapidly in late pregnancy, it is possible to predict the patients who are going to be at risk of RDS at birth. This, of course, is especially so if a mother has previously had a child with RDS so that the subsequent ones are apt to have the same manifestations.

The compound is produced *de novo* by two pathways: the choline incorporation route (I) and phosphatidyl ethanolamine methylation (II). To establish the contribution of each pathway, lung slices have been incubated with pathway specific isotopic precursors, including ^{14}C-choline, ^{14}C-methionine, and ^{14}C-ethanolamine and incorporation of isotope into lecithin was determined. Measurement of precursor uptake was also performed, and the phospholipid synthesis rates were adjusted for isotope transport.

		LECITHIN SYNTHESIS (cpm/mg lung/hr)	
Gestation	No.	Pathway I	Pathway II
102 days	1	246	11
130 "	2	517	25
144 "	6	470	8
150 "	5	927	13
Neonates (24-72 hr old)	5	375	16

The major pathway in all animals examined is the choline incorporation route. Whereas II shows a relatively constant, low rate throughout gestation (term 164 days), I increases at 150 days gestation ($p<.05$) and then decreases in the neonate.

It is concluded that pathway I is predominant and accounts for increased lecithin synthesis in the mature fetus as well as for the surge in amniotic fluid lecithin/sphingomyelin ratio. (Dr. P. Farrell, Dr. D. Lundgren (guest worker), NIAMDD; Dr. M. Epstein, NICHD).

Lecithin synthesis in fetal primate tissues as measured *in vivo:* This *in vivo* study examined the incorporation of ^{3}H-choline and ^{14}C-ethanolamine

into lecithin. Isotopes were injected into 140-148 day fetal Rhesus monkeys via an interplacental venous catheter maintaining the integrity of the amniotic cavity and the circulation. After 2 hrs, $^{3}H/^{14}C$ labeling in TLC-isolated lecithin was measured in various organs. Results (% transported precursor incorporated into lecithin) from 4 fetuses indicate that pathway I predominates over pathway II

LECITHIN SYNTHESIS	Lung	Liver	Brain	Kidney
Pathway I	82	43	88	70
Pathway II	1.7	4.4	5.2	1.3

(phosphatidyl ethanolamine methylation) in these tissues. ^{3}H-lecithin was present in fetal plasma after 2 hrs but not in tracheal or amniotic fluid. Maternal treatment with IM betamethasone 48 hrs prior to delivery resulted in greater than normal incorporation of ^{3}H-choline into lecithin in lung, but not liver, brain, or kidney in 2 fetuses examined thus far. Additionally, one acidotic fetus showed a reduced pathway I rate in all organs except brain. *Therefore*, the choline incorporation pathway of fetal primate lung: a) is predominant in synthesis of lecithin, and b) may be enhanced by corticosteroids and depressed by acidosis. (Dr. P. Farrell, E. Knight (guest worker), NIAMDD; Dr. M. Epstein, Dr. A. Fleischman, Dr. G. Oakes, NICHD).

Regulation of pulmonary lecithin synthesis in the fetal rat: Regulation of pulmonary lecithin (the principal surfactant phospholipid) synthesis is of crucial importance to the fetus and neonate. In our studies metabolic control mechanisms have been investigated in lung samples from over 700 rat fetuses of varying gestational ages. It has been found by incubation of lung slices with isotopic precursors that the choline incorporation pathway accounts for >97% of lecithin synthesis. The rate of this pathway is significantly ($p<.001$) enhanced after 20 days gestation (term = 22 days). Specific activities of two pathway enzymes, choline kinase (CK) and choline phosphotransferase (CPT), have been measured in lung homogenates from rat fetuses of 16-22 days gestation (DG). CK rises from 740 at 16.5 DG to 1440 pmole/min/mg protein at 19.6 DG ($p<.001$) and falls from this peak value to 760 pmole/min/mg at 21.3 DG. CPT, although rising from 38 pmole/min/mg at 16.5 DG to 54 at 19.6 DG ($p<.02$), does not peak until 20.5 DG (60 pmole/min/mg, $p<.001$). The enzyme then declines in activity as the fetus approaches term. Kinetic properties (Km values, pH profiles, etc.) have also been determined for CK and CPT.

These findings suggest that CK and CPT may play a key role in accelerating pulmonary lecithin synthesis in the developing fetus. However, since the rate of lecithin production continues to increase after 21 DG when CK and CPT activities are declining, other regulatory mechanisms may be operative in the term fetus and neonate.

We have previously shown that CPT and lecithin synthesis from choline are induced by corticosteroids (Science 179:297, 1973). It is now apparent that: a) fetal lung lecithin is produced primarily by the choline incorporation pathway, and b) enhancement of this pathway occurs in late gestation, following an increase in the activities of CK and CPT. It is probable that pulmonary

lecithin deficiency in RDS is due to insufficient development of pathway I which may in turn be secondary to low CK and CPT activities. (Dr. P. Farrell, Dr. D. Lundgren (guest worker), NIAMDD).

Epidemiology of respiratory distress syndrome: Neonatal mortality due to RDS or hyaline membrane disease (HMD) has never been precisely determined, but merely estimated on the basis of small samples skewed by local factors and not representing the national picture. Due to the long lead time for collection and coding of death certificates, the necessary data for 1968 have only recently become available from the National Center for Health Statistics. These statistics, the first national figures for RDS, were stored on magnetic tapes which were then computer processed by us. Our analysis showed RDS or HMD to be the underlying cause of death in 8273 cases and a major contributing factor in 2724 more, a total of 19.3% of all neonatal deaths. The RDS mortality rates for whites compared to non-whites were 2.30 *vs.* 2.68 per 1000 live births and 32.4 *vs.* 19.5 per 1000 premature (less than 2.5 kg) births, respectively.

RDS deaths were evenly distributed throughout the year. The male/female ratio was 1.76 compared to 1.41 for all neonatal deaths. Age at death conformed to clinical experience: 46% on day 1, 26%-day 2, 15%-day 3, 5%-day 4, and 2% on day 5. Comparison of RDS mortality rates with overall neonatal mortality rates for each state showed general agreement except for a few states in which the RDS rates were significantly different.

These data will be useful in planning regional neonatal intensive care facilities and will provide a baseline for later evaluation on a national basis of newer therapeutic methods. (Dr. R. Wood, Dr. P. Farrell, NIAMDD).

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Metabolic, Physiological, and Biochemical Studies in Cystic Fibrosis.

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Philip M. Farrell, M.D., Ph.D., Robert E. Wood, M.D., Ph.D., David W. Lundgren, Ph.D., Michael Klagsbrun, Ph.D., John G. Bieri, Ph.D., NIAMDD; Joseph C. Fratantoni, M.D., CC.

Cooperating Units: Laboratory of Biochemical Pharmacology, NIAMDD; Laboratory of Nutrition and Endocrinology, NIAMDD; Clinical Pathology Department, CC; Vernon Fischer, Ph.D., St. Louis University Medical School; Samuel S. Spicer, M.D., Medical College of S. Carolina, Charleston.

Man Years:

Total:	4
Professional:	1.5
Other:	2.5

Project Description:

Objectives:

1. Studies in tissue culture fibroblasts: Although the primary biochemical error in cystic fibrosis (CF) remains unknown, several lines of evidence suggest that fibroblasts from CF patients are metabolically abnormal. It has been reported that fibroblasts in tissue culture from patients with CF show decreased methylation of total RNA. In addition, it has been claimed that fibroblasts from obligate heterozygotes with CF also manifest reduced RNA methylation to values intermediate between controls and CF homozygotes. Such an alteration might have important metabolic implications. Therefore, RNA methylation has been studied in CF fibroblasts by applying improved techniques of cell culture, RNA labeling, and RNA isolation and fractionation.

2. Effects of vitamin E deficiency in man: The biological manifestations of vitamin E deficiency are more protean in various animal species than for any other nutrient. However, man has not been shown conclusively to develop clinical symptoms of vitamin E deficiency. Patients with CF represent the ideal experimental model of profound vitamin E deficiency, often present for

many years. Study with sophisticated methods in a comprehensive approach is permitting a better definition of the changes in metabolism which are produced by a deficiency in this vitamin.

3. Blood polyamines in cystic fibrosis patients and CF heterozygotes: It has been suggested that blood polyamine levels may be altered in CF homo- and heterozygotes. If such an alteration were consistently demonstrable in the latter group, measurement of polyamines might serve as a CF carrier detection test. Accordingly, we have undertaken an evaluation of polyamine metabolism in CF by initially measuring blood spermine (Sp) and its precursor, spermidine (Spd) in CF patients and obligate heterozygotes.

4. Biochemical assessment of ciliary activity in the presence of cystic fibrosis serum: Since Spock first observed that serum from CF patients and heterozygotes induced ciliary dysrhythmia, several "CF factor" bioassays have been described. Such methods, however, suffer from several disadvantages in that they are: a) subjective, b) qualitative, and c) difficult to reproduce. We have had consistently negative experiences with both rabbit tracheal and oyster gill assays. In an effort to develop a quantitative chemical assay, we have tried a fresh approach by measuring motility-coupled ciliary ATP hydrolysis in the presence of serum.

Methods Employed:

1. Control and CF fibroblasts were cultured in the presence of L-(methyl-^{14}C) methionine and 5-^{3}H uridine. RNA was then extracted and fractionated by sodium dodecyl sulfate sucrose density gradient centrifugation. Ratios of ^{14}C/^{3}H incorporation serve as a measure of the extent of RNA methylation. In addition, methylated base composition of human fibroblast tRNA was identified by two-dimensional chromatography. Methylation rates were also measured *in vitro* by incubating fibroblast extracts with E. *coli* tRNA and either S-adenosyl (methyl-^{3}H) methionine or S-adenosyl (methyl-^{14}C) methionine.

2. Vitamin E as the abundant α-tocopherol isomer has been measured spectrophotometrically after isolation by thin layer chromatography. Erythrocyte hemolysis has been measured after addition of hydrogen peroxide to washed RBC suspensions and RBC survival has been determined with ^{51}Cr-tagged cells. Creatine excretion has been determined utilizing a specific enzymatic technique for the metabolite. Skeletal muscle has been examined histologically and histochemically and jejunal mucosa has been ultrastructurally studied with standard electron microscopic techniques. Various muscle-derived plasma enzymes, e.g., creatine kinase, have been determined by standard techniques.

3. Using plastic containers wherever possible to maximize recovery, citrated whole blood was extracted twice with 3% perchloric acid. Subsequently, polyamines were concentrated by lyophilization, separated by ion exchange chromatography, and quantitated with ninhydrin using an amino acid analyzer. Utilizing these techniques, consistent recoveries (68 ± 1%, mean ± SEM, N = 6) of spermidine and spermine were obtained as determined by addition of ^{14}C-labeled compounds.

4. To characterize the ATPase activity several basic studies were performed with oyster gill and rabbit tracheal cilia. Homogeneous, motile cilia were prepared from the former by mincing in 0.05 M Tris HCl (pH 8.0), 0.3 mM EDTA. The effect of various factors (time, temperature, pH, cations, and substrate concentration) on ciliary ATP hydrolysis was determined. Explants, homogenates, and minces of rabbit trachea and oyster gill were preincubated for 30-45 min with either normal, CF, or CF heterozygote sera; subsequently ATP-γ-^{32}P was added and ^{32}Pi production was measured.

Major Findings:

1. Examination of $^{14}C/^{3}H$ incorporation revealed that tRNA, 18S rRNA, and 28S rRNA of CF cells were methylated to a normal extent. Methylated base composition was found to be the same in tRNA and rRNA for CF and control cells. Furthermore, this pattern was identical to that previously found for RNA from yeast, various animal tissues, and HeLa cells. Measurement of methylation *in vitro* revealed statistically unaltered rates for CF fibroblast extracts. Identification of the bases again revealed a normal pattern for CF cells.

2. Cystic fibrosis patients with pancreatic achylia have uniformly shown decreased plasma α-tocopherol. Abnormal erythrocyte hemolysis *in vitro* has been found in all individuals with less than 400 µg/100 ml α-tocopherol. Excessive hemolysis correlates in a sigmoid fashion with plasma α-tocopherol. Determination of ^{51}Cr-RBC survival revealed a mean $t_{1/2}$ of 22 days in 19 deficient patients, with 3 subjects particularly low at <18 days; the average $t_{1/2}$ in 4 CF patients after α-T supplementation increased from 21 to 26 days. Muscle involvement has been assessed by measuring creatine excretion and the plasma activity of muscle derived enzymes. Creatinuria was present in 13 of 25 patients studied, with the total group of 25 showing an average creatine excretion of 200 mg/24 hr (normal mean = 20 mg/24 hr, $p<.01$). Elevated plasma aldolase and CPK (the skeletal muscle isozyme) activities have been found in 2 subjects thus far. Subcellular membranous structures, examined electron microscopically in intestinal biopsy samples, were found to be normal in α-T deficiency.

3. The data indicate that CF homo- and heterozygote blood generally shows increased Spd and decreased Sp leading to significantly altered Spd/Sp ratios: Control - 1.06, CF - 1.48, heterozygote - 1.73. Eight of the ten CF heterozygote samples were beyond the 95% confidence interval of a control group and the CF heterozygote samples as a whole were significantly different ($p<.05$) than the total group of 13 controls.

4. The preparation of homogeneous, motile cilia on electron microscopy showed intact cilia and was found to hydrolyze ATP in proportion to calculated beat frequency. A high molecular weight ATPase was purified for the first time from rabbit tracheal cilia and shown to have properties similar to flagellar dynein. Explants, homogenates, and minces of rabbit trachea and oyster gill, preincubated for 30-45 min with either normal, CF, or CF heterozygote sera were found to hydrolyze ATP normally, i.e., the latter two groups could not be distinguished from normal serum.

Significance to Biomedical Research:

1. These results indicate that all tRNA methylase enzymes are fully active in CF fibroblasts. The extent of tRNA and rRNA methylation is unaltered in CF and there are no differences in methylated base composition. Application of these improved techniques over those used by other investigators has shown that decreased RNA methylation is not a fundamental feature of CF fibroblasts, if present at all. Measurement of RNA methylation in CF fibroblasts and lymphocytes should not be employed as a means of carrier detection.

2. These investigations which are still in course indicate so far that vitamin E deficiency in man is associated with many of the findings present in animals, but generally at a subclinical level. Such findings are: 1) Hemolysis, both *in vitro* and *in vivo* which can be corrected with administration of vitamin E or the addition of tocopherol to the *in vitro* system. 2) Evidence of muscle involvement as shown by creatinuria, occasional elevation of plasma activities of muscle derived enzymes, and deposition of ceroid pigment in smooth muscle.

The metabolic role of this ubiquitous biological antioxidant is still unclear, but its widespread presence in human tissues must serve a purpose. Patients with malabsorption and secondary tocopherol deficiency should, therefore, receive supplements of vitamin E. Such supplementation should also be considered in the dietary makeup of populations in areas of the world (e.g., Bangladesh, Jordan, etc.) suffering from malnutrition and receiving inadequate diets. However, there is little reason to add vitamin E supplements in countries such as the United States where people ingest adequate amounts of the vitamin in their diets and have normal blood tocopherol levels.

3. Findings thus far suggest that blood levels of polyamines are disturbed in cystic fibrosis homozygotes and heterozygotes. Further study will be required to determine if this apparent abnormality will be useful in population screening for carriers of the CF genotype. In addition, in the view of the widespread distribution of polyamines in cells and tissues as well as their probable abnormality in tissue cultured fibroblasts, further investigations may provide an insight as to the nature of the elusive basic defect in cystic fibrosis.

4. In this project, a new assay for ciliary motility based on ATP hydrolysis, has been developed and for the first time a ciliary ATPase, similar to flagellar dynein, has been isolated and partially characterized. Measurement of ciliary ATPase activity and utilization of ATP by motile cilia in the presence of CF sera has revealed no differences as compared with assays in the presence of normal sera. Studies are continuing but this objective, quantitative assay has thus far failed to demonstrate the "CF factor."

Honors and Awards: None

Publications: Klagsbrun, M., and Farrell, P.M.: The methylation of transfer and ribosomal RNA in human fibroblasts: Normal methylation of RNA in cystic fibrosis. Pediat. Res. 8:205-211, 1974.

Serial No. NIAMDD-PMB-2c
1. Pediatric Metabolism
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Clinical Studies in Cystic Fibrosis

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Robert E. Wood, M.D., Ph.D., Philip M. Farrell, M.D., Ph.D., NIAMDD; Herbert Y. Reynolds, M.D., NIAID.

Cooperating Units: Laboratory of Clinical Investigation, NIAID.

Man Years: Total: 2.5
Professional: 1.5
Other: 1.0

Project Description:

Objectives:

1. A study of Pseudomonas vaccine in cystic fibrosis (CF): To measure antibody responses to pulmonary colonization and infection with Pseudomonas species and to immunization with a vaccine prepared against Pseudomonas aeruginosa. To demonstrate safety and possible clinical efficacy of Pseudomonas vaccine in patients with CF.

2. Tracheal mucociliary transport in cystic fibrosis: To measure the rate of mucociliary airway clearance in patients with CF by a newly developed direct technique; to study the relationship of mucociliary airway clearance rates to clinical disease in CF; to evaluate the effect of pharmacological agents on mucociliary airway clearance as a possible therapeutic method.

3. Epidemiology of cystic fibrosis in the United States, 1960 through 1970: To analyze the patterns of mortality due to CF; to assess the effects of specialized care on the mortality in CF.

Methods Employed:

1. Patients with CF were given a series of injections of Pseudomonas vaccine. Antibody titers were measured in serum and bronchial secretions. A small series of patients were also given Pseudomonas vaccine by intranasal spray. Antibody titers were measured before and after immunization in serum,

parotid saliva and bronchial secretions. Patients were monitored for clinical and immunological responses.

2. Adult patients with cystic fibrosis were studied. A fiberoptic bronchoscope was passed into the trachea under topical anesthesia. Teflon discs of calibrated size were blown onto the tracheal mucosa and their movements filmed with a movie camera. From analysis of the change in image size of each Teflon disc with time, the rate of motion was calculated. Following the initial measurement of mucus velocity patients were injected with 0.25 mg of terbutaline and their mucus velocities measured again 15 minutes later.

3. Data were obtained from the National Center for Health Statistics and from the Data Registry of the National Cystic Fibrosis Research Foundation on the number of deaths in each age group for the years 1960 through 1970. Statistical analysis of this data was then carried out.

Major Findings:

1. Pseudomonas vaccine is well tolerated by patients with CF whether given by parenteral or intranasal routes. Antibody levels rise significantly following injection. Evaluation of response to intranasal vaccine is pending at the time of preparation of this report. Sample size at present is too small to meaningfully evaluate clinical response.

2. The average rate of tracheal mucus velocity in 14 patients with CF was approximately 15% that of 20 normal volunteers of the same age. In response to a single injection of terbutaline, a beta-adrenergic agent, the average mucus velocity increased to more than twice the control rate in patients with CF but not in normal volunteers.

3. During the decade 1960 through 1970 there were approximately 625 deaths due to CF in the United States each year. Comparison of mortality experience in patients followed by Cystic Fibrosis Research, Care, and Teaching Centers with the patients not followed in such Centers suggests strongly that intensive and vigorous care results in greatly prolonged survival with an average age at death some ten years older in patients followed by the CF Centers.

Significance to Biomedical Research:

1. Most patients with CF have chronic pulmonary colonization with Pseudomonas which is an organism notoriously resistant to most antibiotics. The development of an effective immunization program against Pseudomonas species could be of significant therapeutic benefit to these patients.

2. This is the first demonstration of pharmacological increases in mucociliary clearance in CF. While the achieved rates of mucus transport following the injection of terbutaline are still only one-third of normal, we believe that further increases may be possible by different drugs, routes of admini-

stration, or drug combinations. This observation has great potential benefit in the therapy of patients with the chronic obstructive lung disease of CF.

3. Many physicians feel that the prognosis in CF is essentially hopeless and therefore do not aggressively pursue therapy. This study strongly suggests that vigorous therapy does significantly increase longevity and that care in a specialized Cystic Fibrosis Center is an effective means of improving the prognosis in individual cases of cystic fibrosis.

Honors and Awards: None

Publications: Pennington, J.E., Reynolds, H.Y., Wood, R.E., Robinson, R.A., and Levine, A.S.: Use of a Pseudomonas aeruginosa vaccine in patients with acute leukemia and cystic fibrosis. Am. J. Med., 1974 (in press).

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Pulmonary Lecithin Synthesis as Related to the Respiratory Distress Syndrome

Previous Serial No.: None

Principal Investigator: Paul A. di Sant'Agnese, M.D.

Other Investigators: Philip M. Farrell, M.D., Ph.D., Robert E. Wood, M.D., Ph.D., David W. Lundgren, Ph.D., NIAMDD; Ronald A. Chez, M.D., Michael F. Epstein, M.D., T. Allen Merritt, M.D., NICHD

Cooperating Units: Pregnancy Research Branch, NICHD; W. Blackburn, M.D., University of South Alabama, Mobile.

Man Years: Total: 3.5
Professional: 1
Other: 2.5

Project Description:

Objectives:

Respiratory distress syndrome (RDS) or Hyaline membrane disease occurs primarily in premature newborns and is characterized by progressive pulmonary atelectasis. The disorder has a higher incidence than any other pediatric pulmonary disease of metabolic origin. It has become quite clear in the last several years that a decrease in the concentration of lung surfactant (composed primarily of disaturated lecithin) is causally related to RDS. By implication, a decreased rate of pulmonary lecithin synthesis is the likely pathogenetic factor in the disease. Although the etiology of the disorder is not known, a metabolic error (inborn error of metabolism) in lecithin synthesis must be highly suspect. In order to avoid neonatal atelectasis, it is essential for the fetal lung in late gestation to increase its capability for lecithin synthesis and elevate the concentration of intracellularly stored phospholipids. Thus, prior to birth, lecithin synthesis in fetal lung must be carefully controlled such that acceleration is initiated at the proper time in gestation. A rational therapeutic approach, therefore, must be aimed at increasing the rate of surfactant (lecithin) synthesis. The objectives of this project are as follows: The principal *de novo* pathway for lung lecithin synthesis will be determined by measuring the rate of lecithin formation from either choline incorporation or phosphatidylethanolamine methylation. Since some investigators

have claimed that pronounced species specificity occurs in regard to the relative contributions of the two pathways, several species (both adult and fetus) are being examined, including human, monkey, rabbit, and rat. The key question of what factors regulate lung lecithin synthesis is being approached by studies on the enzymes of the two pathways and by determining levels of substrate (metabolites) in the pathways. By determining the changes in rate of lecithin synthesis as a function of gestation, the importance of regulatory factors will be determined. Conversion of choline to the phosphoglyceride involves three enzymatic reactions: 1) phosphorylation of choline, 2) activation of phosphorylcholine to the CDP-choline intermediate, and 3) incorporation of phosphorylcholine into 1,2-diacylglycerol to form lecithin. It seems likely that the rate of one or more of these reactions increases to elevate the flux of choline in developing fetal lung. Since modulation of reaction rates is achieved mainly by control of enzymes catalyzing key reactions, this hypothesis has been examined by initially determining choline kinase and choline phosphotransferase activities in developing fetal rat lung. In addition, the role of corticosteroids in controlling lung maturation and lung lecithin synthesis is being assessed in both fetal and newborn animals.

Methods Employed:

Lecithin synthesis is measured by thin layer chromatography and by isotopic incorporation studies with lecithin precursors. The latter technique has been applied both *in vitro* with lung slices and *in vivo* with fetal Rhesus monkeys injected *in situ* with ^{3}H-choline and ^{14}C-ethanolamine. Activities of the enzymes in the choline incorporation pathway (choline kinase, cytidyl transferase, and choline phosphotransferase are measured by newly developed techniques using isotopically labeled substrates. Rabbit, rat, and monkey fetuses have been injected with corticosteroids *in utero* and followed by various periods of time with respect to lecithin synthesis and enzyme activities. Additionally, decapitated rat fetuses, corticosterone deficient and low in lung lecithin, have been utilized as a model of hormone deficiency. Inhibitors of protein synthesis, such as cycloheximide and actinomycin D have been employed in order to define the specific site of action for corticosteroids in fetal lung lecithin synthesis.

Major Findings:

Lung lecithin concentration and isotopically-measured rate of lecithin synthesis increases during the latter 10-20% of gestation for fetal rabbit, rat, and monkey. The principal pathway for lung lecithin synthesis was found to be the choline incorporation route rather than phosphatidylethanolamine methylation. Administration of corticosteroids to the fetus enhances the rate of lung synthesis within six hours after injection and increases the activity of choline phosphotransferase (CPT). These changes may be blocked by prior administration of cycloheximide but not actinomycin D. The latter data suggest that corticosteroids act to promote mRNA translation for CPT rather than DNA transcription. Corticosterone deficient decapitated rat fetuses show low lung choline phosphotransferase activity which may be increased to normal within six hours after a physiological dose of dexamethasone.

In normally developing fetal rats, measurement of lung choline kinase and choline phosphotransferase as a function of gestational age revealed that the former significantly rises at 19-20 days and the latter increases to a peak value at 20-21 days. These enhanced enzyme activities develop within 24 hours after the reported elevation of fetal plasma corticosterone and precede the change in rate of lecithin synthesis by 24-48 hours.

Various kinetic properties of the three enzymes in the choline incorporation pathway have been determined and choline kinase has been partially purified. By full order of magnitude, choline phosphotransferase possesses the lowest specific activity of the three enzymes, and thus appears to be the rate limiting catalyst in the pathway. Choline kinase shows atypical, sigmoidal kinetics with variable choline concentrations suggesting the possibility of allosteric modulation.

Significance to Biomedical Research:

Prior to 1971, despite the overwhelming importance of respiratory distress syndrome as a community health problem, very little was known in general about lung biochemistry and in particular about the synthesis of surface active lecithin by fetal and newborn lungs. Application of fundamental biochemical techniques to this problem is the most reliable approach and thus far has been quite fruitful. In order to identify the metabolic error responsible for RDS, basic features of the lung lecithin pathway must be determined, including those factors which regulate pathway rate. These studies have thus far indicated that corticosteroids are powerful inducers of lung lecithin synthesis, an action mediated by enzyme (choline phosphotransferase - CPT) induction. This enzyme and choline kinase exhibit many of the features characteristic of key catalysts in a metabolic sequence. Results for fetal rat lung suggest that choline kinase and choline phosphotransferase are important in the initiation of enhanced lecithin synthesis through the choline pathway. Thus, these enzymes may play a role in the etiology of RDS.

Honors and Awards: None

Publications:

Farrell, P.M.: Regulation of pulmonary lecithin synthesis. In Villee, C.A., Jr., Villee, D.B., and Zuckerman, J.E. (eds.): Respiratory Distress Syndrome. New York, Academic Press, 1973, pp 311-341.

Farrell, P.M., Lundgren, D.W., and Adams, A.J.: Choline kinase and choline phosphotransferase in developing fetal rat lung. Biochem. Biophys. Res. Comm. 57:696-701, 1974.

I. Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis (Drs. Shulman, Gordon, and Barber

1. Molecular Characteristics of Purified Factor VIII.

We have developed methods for obtaining highly purified Factor VIII, (the factor that is deficient in classical hemophilia) utilizing limited chymotrypsin digestion of crude F VIII concentrates and large-pore gel filtration. The purified factor has a molecular weight greater than 2 million but after sulfhydryl reduction, dissociation occurs into major subunits of 240,000 MW. Other investigators have found that purified F VIII can be dissociated into high and low molecular weight components by high ionic strength or detergents. We have observed that physiologic recalcification of citrated normal human plasma or cryoprecipitate (a crude concentrate of F VIII) which causes spontaneous clotting of the plasma or concentrate, produces two components of F VIII that are similar in size to the components obtained by other dissociating agents. The low MW component has procoagulant activity (i.e. initiates clotting of hemophilic blood) but the high MW component does not. The coagulant effect of the low MW component is inhibited by anti-F VIII antibodies made in rabbits or arising spontaneously in human beings, but these antibodies do not produce positive immunoprecipitation or radioimmunoassay with the low MW components. The high MW component is precipitated and detected in radioimmunoassay by rabbit anti-F VIII. Thrombin, in amounts approximately equal to those that form on recalcification of plasma or cryoprecipitate, when added directly to purified F VIII, causes separation of F VIII into high and low MW components that have the same attributes as those formed during physiologic recalcification. The components produced from purified F VIII by physiologic recalcification or thrombin did not recombine when incubated together. Thus, F VIII appears to be dissociated irreversibly by intrinsic clotting into high and low MW components which are functionally distinct yet antigenically related. Thrombin, a product of intrinsic clotting, appears to have a role in the generation of low MW F VIII. Current studies are underway to clarify the relationship between F VIII components obtained by clotting and those obtained otherwise, and to determine whether the high MW component obtained as we described is an adsorbant of, rather than an integral part of, F VIII and/or the von Willebrand factor involved in platelet function.

2. Platelet Adenylate and Guanylate Cyclase

In our studies last year we found that agents that increase cyclic AMP inhibit metabolic responses of platelets and prevent the release reaction and aggregation whereas agents that decrease cyclic AMP initiate release and aggregation. Since reports in the literature indicate that most other cells show increases in cyclic AMP with stimulation of cell function it was of interest to see whether guanylate cyclase and cyclic GMP might change in platelets in the same manner as adenylate cyclase and cyclic AMP do in other cells when they are stimulated. Results obtained on studies of cyclic GMP changes in platelets were equivocal because the most sensitive methods available for measuring this compound (radioimmunoassay) did not show clear differences in

levels of cyclic GMP between resting and stimulated platelets. However it was found that guanylate cyclase increased two-fold or more in platelets stimulated by aggregating agents compared to controls. In this respect platelets respond to aggregating agents like heart muscle to agents that depress cardiac contractility, i.e. by an increase in guanylate cyclase and a simultaneous decrease in adenylate cyclase. Currently studies are underway to determine how the hormone-like prostaglandins and their metabolic precursors, which markedly affect platelet aggregation and metabolism, influence the level of platelet cyclic nucleotides.

3. Subcellular Localization of Adenylate Cyclase and Cyclic 3',5'-Nucleotide Phosphodiesterase in Human Blood Platelets

The distribution and recovery of adenylate cyclase and cyclic 3',5'-nucleotide phosphodiesterase was determined in subcellular fractions of human blood platelets. Adenylate cyclase was firmly associated with the plasma membrane fraction and none was in the soluble fraction. The specific activity of adenylate cyclase was approximately the same in the granule-rich fraction as in the whole cell lysate. Phosphodiesterase activity was found in the soluble fraction, with approximately 96% of the activity recovered in this fraction and less than 1% in the membrane fraction. Only 3% of the platelet phosphodiesterase activity and none of the adenylate cyclase activity was released in a soluble form after thrombin treatment of intact platelets. Following thrombin treatment of intact platelets over 92% of the total phosphodiesterase was recovered whereas only 58% of the total adenylate cyclase was recovered. These findings indicate that a large amount of the platelets adenylate cyclase is inactivated following thrombin treatment. Compartmentalization of adenylate cyclase tightly bound to membranes and phosphodiesterase in the soluble cytoplasmic fraction helps explain the difficulties encountered in correlating total cellular cyclic nucleotide levels with platelet function particularly in reactions between platelets and aggregating agents such as antibodies which disrupt platelet structure.

II. Study of the Immunology of Blood Cell Deficiencies (N. R. Shulman and N. Gordon)

1. Isoimmune Platelet Disorders

In the past few years, through numerous referrals we have had an opportunity to evaluate the significance of specific platelet isoantigens as the cause of neonatal isoimmune purpura and post-transfusion purpura. In the study of approximately 200 neonatal purpura cases we have found that approximately 50% can be established as being isoimmune in origin based on measurement of antibody in maternal serum and phenotyping parental platelets. One specific platelet antigen that we first identified in 1959, the $P1^{A1}$ antigen, has been found to be responsible for most cases of isoimmune neonatal purpura. Although this antigen is lacking in only 2% of the population and therefore mismatched in approximately 2% of marriages it accounts for more than half of all suspected instances of isoimmune neonatal purpura. This antigen therefore appears to be the most antigenic of all platelet antigens, a fact in keeping with the observation that first born children are as likely to be affected as subsequent infants in contrast to the circumstance in Rh sensitization where the first

born is rarely affected. Evaluation of different forms of treatment including exchange transfusion, steriod therapy and massive platelet transfusion suggests that the latter treatment is the simplest and most effective. It has been found possible to use antigenic platelets in therapy of the new born because the antigen-antibody reaction, aside from destroying platelets, produces no systemic response. Hence antigenic platelets can be utilized to adsorb antibody which is removed by platelet sequestration, allowing the infants own platelets to circulate.

Six referred instances of post-transfusion purpura have been studied serologically with the finding that, in all instances, the sensitizing antigen leading to autologous platelet destruction was Pl^{A1}. Of special interest was the fact some individuals developed agglutinating or blocking antibodies rather than complement fixing antibodies which have been identified in all previous cases. Exchange transfusion in two instances was found to be the most effective form of therapy in keeping with results of our initial reported case. The fact that antibody was present in the exchange patients after recovery adds weight to the hypothesis that post-transfusion purpura is caused not by antibody alone, but by antibody in complex with antigen derived from transfused platelets.

2. Naturally Occurring Anti-Factor VIII

Acquired hemophilia results from development of an antibody against F VIII in previously normal individuals. This disease represents an unusually severe form of "autoimmunity" and reasons for an antibody developing against a circulating protein remain totally obscure. Some reports in the literature suggest that acquired hemophilia results from malignant mutation and have presented evidence that the anti-F VIII is monoclonal. Our results conflict with this point of view in that the distribution of both κ and λ light chains of anti-F VIII in patients we have studied is typical of normal gamma globulin. In attempts to determine the molecular specificity of acquired anti-F VIII, the human antibodies were compared with anti-F VIII elicited in rabbits against purified F VIII. Human and rabbit anti-F VIII have only one attribute in common, the neutralization of F VIII coagulant activity. However, whereas rabbit anti-F VIII precipitates F VIII and agglutinates F VIII-coated erythrocytes, human anti-F VIII does not elicit any secondary detectable immunologic reaction. Moreover, although rabbit anti-F VIII cross-reacts with an apparent inactive molecule in hemophilic blood, human F VIII does not appear to be neutralized by hemophilic material. Both rabbit and human antibodies are neutralized by both high and low MW derivatives of purified F VIII (see Project I). It is hoped by continued analysis of the naturally occurring antibodies that a basis will be found for the unusual sensitization that leads to an "autoimmune" syndrome.

3. Development of Immunologic Diagnostic Tests

We have found that our experience with general hematologic hemagglutination techniques can be utilized to develop diagnostic tests of clinical importance. The technique involves stabilization and chemical activation of erythrocytes by the effects of aldehydes on red cell membranes. Aldehydes, particularly pyruvic aldehyde, preserves the red cell in such a way that it can be lyophilized and is not subject to hypo- or hypertonic lysis; and red cells thus

treated are capable of forming stable complexes with a variety of proteinaceous substances. For example, we have coupled hepatitis virus, toxoplasma extracts, purified antihepatitis antibody, and albumin-morphine conjugates to aldehyde treated red cell as the basis for tests for hepatitis antibody, toxoplasma antibody, hepatitis antigen, and detection of addictive drugs in urine and other body fluids. All of these tests have the accuracy of radioimmunoassays but are simpler to perform and have immediate widespread application in medical and public health practices.

Serial No. NIAMDD-CHB-1c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of Blood Coagulation and Diseases of Hemorrhage and Thrombosis

Previous Serial Number: SAME

Principal Investigator: Dr. N. Raphael Shulman

Other Investigators: Dr. Norman Gordon

Collaborating Investigators: Dr. Alfred Barber, American Cancer Society Fellow.

Man Years
Total: 2
Professional 1
Other: 1

Objectives:

Study of the reactions and interactions of coagulation factors in vitro and in vivo to define further the nature of the blood coagulation mechanism, to determine factors of significance in the pathogenesis of diseases of hemorrhage and thrombosis, and to develop better forms of therapy for these diseases.

Methods Employed:

Methods of protein purification and characterization, techniques of enzymology applied primarily to proteolytic enzymes and their inhibitors, kinetic analyses of enzyme reactions, procedures for quantitative measurement of various clotting factors, pharmacologic and physiologic techniques applied in man and animals, and assessment of metabolic pathways of blood cells with radioactivity labeled substrates.

Publications:

1. R. F. Lange, N. R. Shulman, P. A. Tomasulo, and C. N. Coleman: Metabolic Effects of Antibodies on Platelets.Trans. Assoc. Americ. Phys. LXXXVI, 131 1974.

Serial No. NIAMDD-CHB-2c
1. Clinical Hematology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Study of the Immunology of Blood Cell Deficiencies

Previous Serial Numer: SAME

Prinicipal Investigator: Dr. N. Raphael Shulman

Other Investigators: Dr. Norman Gordon

Man Years
Total: 2
Professional 1
Other: 1

Project Descripition:

Objectives:

To study the nature of immunologic reactions which result in formation of antibodies against autologous blood cells, and to determine the significance of immunity in development of "idiopathic" blood cell deficiency states. Of special interest are the biochemical reactions which result in formation of complexes between cells, antibodies, and drug haptenes and the physiologic processes which result in sequestration of cells with attached antibodies. In recent years our work on identifying inherited leukocyte and platelet isoantigens has led to a study of the significance of these antigens in hetero- and homo-transplantation. Immunologic techniques have been used to develop a number of clinically practical diagnostic tests for certain infectious diseases and drug addiction.

Methods Employed:

Techniques of quantitative immunochemistry, including preparation and physiocochemical characterization of purified antibodies and antigens, microanalyses for nitrogen, histamine, and alkaloid drugs, quantitative measurements of complement fixation, cellular agglutinationand precipitin reactions, immunoelectrophoresis, methods of provoking antibody responses in man and animals, and isotopic and flourescent labeling applied to antigens and antibodies. Tissue culture techniques, red cell agglutination techniques and lymphocyte transformation tests, including radioautography. Methods of hemagglutination involving attachment of various antigens to erythrocyes by chemical coupling.

Publications:

1. Hirschman, R. J., and Shulman, N. R.: The Use of Platelet Serotonin Release as a Sensitive Method for Detecting Anti-Platelet Antibodies and a plasma Anti-Platelet Factor in Patients with Idiopathic Thrombocytopenic Purpura. Brit. Journ. of Haem. 24: 6, 1973.

2. Shulman, N. R., Lange, R. F., Tomasulo, P. A. and Coleman, C. N.: Antibodies and Platelet Membranes. Thrombosis: 54, 261, 1974.

3. Shulman, N. R.: Mechanisms of Immunologic Drugs. Effects on Blood Cells. Drugs and Hematologic Reactions. 29th Hahneman Sympoium. Ed. Dimitrov, N. V. 187, 1974.

The period represented by FY 1974 was an interregnum for the Digestive Diseases and Nutrition cluster, with all of the problems and uncertainties associated with such a state. Dr. Lionel Bernstein, former Associate Director for DDN, and his Special Assistant, Dr. Vida Beaven, had assumed their new positions in the Office of the Assistant Secretary for Health, while the DDN Associate Director-Designate was not expected to assume command until August 1974 at the earliest. As an interim measure, Dr. R. W. Lamont-Havers, NIAMDD Deputy Director, assumed the additional title of Acting Associate Director for DDN; however, credit for holding the cluster together during this period must go to all of the staff of this cluster, each of whom performed in an admirable fashion in a most difficult situation. Recognition should also be made of the continued help and involvement of Dr. Bernstein and particularly of the continuing involvement of Dr. Beaven in giving direction to the DDN evaluation program.

During this period there were two additional major staff changes. Dr. Karl Mason officially assumed a "part-time" status while continuing to work as hard as he had done in the past. Mrs. Marjorie Zukel, former Fellowships Officer, assumed the position of Assistant Nutrition Program Director, replacing Miss Joan Fredericks who became Assistant Program Director in the Arthritis, Orthopedics, and Dermatolology area.

The true contribution of the staff and cluster is reflected in the following reports on its two major components, Digestive Diseases and Nutrition.

The Digestive Diseases Program encompasses research of organ systems associated with the gastrointestinal tract. Research activity is concerned with the basic aspects of the physiology and biochemistry of disease entities. Efforts include studies of the cause, prevention, diagnosis and treatment of diseases of the esophagus, stomach and duodenum, pancreas and salivary glands; inflammatory and metabolic diseases of the liver; diseases of the gallbladder and bile passages; absorptive and secretory diseases of the small and large bowel; colonic and inflammatory bowel diseases; gastrointestinal circulatory disease. Also included in this program area are studies related to the psycho-social aspects of digestive diseases; and research, development and evaluation of techniques, methods, or approaches in the use of endoscopic and other instrumentation for the diagnosis, treatment, and investigation of digestive diseases.

A. Research Grants

A sampling of significant advances that have been made under the research grant program during the past year are summarized below:

Very effective histamine antagonists are known which block two of the three major sites of histamine stimulation, namely the vascular system and smooth muscles. Until the past few years, however, no compound was effective in blocking the third site of histamine stimulation, the gastric glands. Very recently, studies have shown that a new antihistamine, Metiamide, seems specific for this action and suggests a highly promising approach to the medical treatment of duodenal ulcer. Research groups have tested the compound in double blind trials in small groups of patients and show it to be highly significant in inhibiting both basal and food-stimulated acid secretion in patients with duodenal ulcer.

Because of certain epidemiological characteristics of Crohn's disease (one of the inflammatory bowel diseases), a transmissible agent is being sought as the etiological agent. One research group is trying to isolate a mycobacterial antigen since patients with Crohn's disease show a greater sensitivity than controls to these agents. Another group has very exciting new data that raises hope that the causative factor may be viral since an agent has been cultured from intestinal tissue of Crohn's disease patients and passed through seven subcul subcultures. These subcultures have continued to show the same cytopathic effect as the original culture. Although this is encouraging data, much more characterization needs to be done before a viral etiology can be ascertained.

Technological advances are often the necessary prerequisite for the understanding of basic physiological processes. Hopefully the new technique of endoscopic retrograde pancreatography will allow more basic information to be collected about the exocrine pancreas and its function in health and disease. Only five percent of our active grants are direct to studies of the pancreas. With this promising new technological advance, which allows the visualization of the tract and collection of pure pancreatic fluid without surgery, more research may be undertaken to provide the answers to the many unanswered questions in this area.

Although one-fifth of the active grants in the digestive diseases program are directed to the study of liver disease, advances have been few. However, in this past year a significant achievement has occurred. It is now possible to produce the entire spectrum of alcoholic liver disease in an animal model, the baboon. This accomplishment is of major importance because it is the first time that the progression from fatty liver through hepatitis to cirrhosis has been conclusively shown and thus clearly confirms what has long been speculated but never established - that alcoholic fatty liver is the precursor of cirrhosis. Moreover, the data suggests that proper nutrition will not prevent the development of alcoholic hepatitis and cirrhosis when excessive alcohol is chronically consumed.

In infants, a rapid diagnosis is often needed to distinguish between two types of jaundice which are unrelated to defective bilirubin metabolism. In the case of extrahepatic biliary atresia, surgery may be indicated, whereas in cholestatic syndrome with patent bile ducts, it may be unnecessary. By feeding the infant cholestyramine, a bile acid absorbing material, it is now possible to obtain a differential diagnosis of the two types of jaundice without exposing the infant to unnecessary surgery.

B. Collaborative Research and Contracts

Two major cooperative clinical trials are now being supported by contracts. The National Cooperative Crohn's Disease Study involving fourteen major research centers are evaluating the therapeutic and prophylactic value of three current drug treatments in regional enteritis. This study is in its second year and nearly 300 patients have been accessioned. It is anticipated that two more years will be required to obtain sufficient data to provide significant information. A second clinical study is in its first year of planning and organization. The National Cooperative Gallstone Study, involving ten study centers, will test the efficacy and safety of orally administered chenodeoxycholic acid in dissolving cholesterol gallstones in human volunteers with asymptomatic radiolucent gallstones. During Phase I, which precedes the double blind clinical trial, the following efforts will be completed: 1) formulation of standards of test drug purity and analysis procedures; 2) initiation of animal toxicology studies required to obtain an IND from the Food and Drug Administration; 3) selection of the test drug supplier for the animal and human studies; 4) selection of the study centers; 5) selection of ancillary research studies; 6) finalization of study protocol; and 7) completion of all procedure manuals and standardization tests. When initiated, the clinical trial will study approximately 1,000 patients over a two year period of drug administration.

An evaluation effort to determine future needs for research in digestive diseases is being currently supported by the Digestive Diseases Program. This evaluation will consist of a series of papers prepared by scientists actively engaged in twelve areas of research in digestive diseases. Currently, the individual reports are being prepared in final form. Following thereafter, three action reports will be prepared. One report will be published in the open medical literature and will serve to alert investigators in the United States to the important areas of research that need additional emphasis. Another report will be for the use of the staff of NIAMDD and its consultants for planning purposes. This brief, but concise, summary document will attempt to assign priorities to major items

of research requiring emphasis and support in digestive diseases. The final report will be a lay document for the public and congressional use and will attempt to interrelate the scientific facts with the public importance of digestive diseases.

In recognition of the need for advanced or novel instrumentation for the diagnosis and treatment of digestive diseases, the Digestive Diseases Program has initiated action to support, on contract, research and development studies in gastrointestinal endoscopy. More specifically, the areas of interest are: a) investigation and development of techniques for the control of gastrointestinal bleeding; b) investigation and development of techniques to facilitate the rapid endoscopic examination of the whole length of the gastrointestinal tract; c) investigation and development of (1) techniques for cannulation and external drainage of the biliary tract and/or pancreas in conjunction with endoscopic retrograde cholangiopancreatography, and (2) new methods to treat pancreatic and biliary disease using the duodenoscope.

08 - Nutrition Program Area

The Nutrition Program of NIAMDD, primarily concerned with support of research directed toward the improvement of human health and longevity, has undergone modest expansion and has made notable contributions relative to its objectives during the past fiscal year.

Developments and Trends in Supported Research

1. Obesity Centers: A major accomplishment of the past year was the establishment of an Obesity Center at St. Luke's Hospital, affiliated with the Medical School of Columbia University. Of four applications received for such centers only two were approved, and only this one justified funding. While support provides for a relatively small nucleus, it is anticipated that as individual research projects are built around this nucleus, and collaboration established with other institutions in the New York City area, a highly visible and important Obesity Center will come into being. In addition, the Nutrition area continues to support a large number of research projects concerned with neurogenic and other factors controlling appetite, feeding behavior, food intake and weight regulation, all of which contribute significantly to this subject of major interest.

2. Fat-Soluble Vitamins: Rather generous support continues to be given to research on 1, 25-dihydroxycholecalciferol, the most active metabolite of vitamin D3, concerned with improved methods of synthesis, its role in absorption and deposition of calcium, and its clinical use in the treatment of uremia and disorders of calcium homeostasis. The efficacy of several other closely related metabolites, more easily prepared synthetically, is under intensive study. Results have been gratifying, and prospects for more effective clinical application of basic findings are most encouraging. Significant progress has been made in identifying intermediary metabolites of vitamin A which may, in the course of time, be of biological and clinical significance comparable to those of vitamin D. Other studies reveal that vitamin A regulates liver secretion rather than synthesis of retinol-binding protein (RBP), so important in vitamin A transport, and that plasma prealbumin (PA) has four binding sites for RBP. Furthermore, the complete amino acid sequences of human PA have been determined. Regarding vitamin E, a recent grantee who happens to have no professional degree beyond the B. Sc. presents evidence that, at least in the rat, the subcutaneous tissue represent a major and previously unrecognized storage site, and the related sebaceous glands of the skin, a major excretory organ, for this vitamin.

3. Minerals and Trace Elements in Nutrition: Of the trace elements, zinc, selenium, chromium and silicon have been in the forefront. A grantee presents experimental evidence that dietary zinc is necessary for mobilzation of vitamin A stores in the liver, in maintaining normal levels of vitamin A in the plasma, and in the synthesis of retinol-binding protein involved in transport of vitamin A. Clinical studies carried out in the Denver area reveal an interesting correlation between low hair zinc levels and poor growth, poor appetite and impaired taste acuity in young and adolescent children. Whether suboptimal zinc nutrition is common in growing children in this country remains to be established.

4. Proteins and Amino Acids in Nutrition: Considerable support has been given to determination of human requirements for amino acids for maintenance of normal nitrogen balance. In a classic study, with himself the subject, a grantee nutritionist has proven convincing evidence that adult man can synthesize his normal requirements for the amino acid histidine, the non-essential nature of which has been questioned in recent years.

5. Parenteral Therapy: Parenteral nutrition, which has high priority in the nutrition area, received only modest support. One grantee reports that I.V. infusates of amino acids free of glucose significantly reduce protein losses and support protein synthesis much more efficiently than the usual therapy with I.V. dextrose. In this context it is noted that a broadly conceived program proposal on parenteral nutrition will, hopefully, be presented to the November 1974 Council.

6. Support of Research Projects (R01 and R22 Grants): Non-competing research grants funded during Fiscal '74 numbered ninety three, as compared to eighty one and seventy five for the two previous years. Five new and two competing grants were activated through use of impounded Fiscal '73 funds. Of the sixty four grant applications (AM primary) approved by Councils for Fiscal '74 funding, thirty were funded (i.e. 47 per cent). In addition, two grants from area 5 and one from area 6 were incorporated in the Nutrition Program and funded.

7. US-Japan Cooperative Medical Science Program: The activities of the Malnutrition Panel of this program were reviewed by the Joint Committee, given a high rating of approval, and authorized to be continued. The number of research grants supported was reduced to eleven during the fiscal year, but it is anticipated that renewal and new applications will bring new and provocative research proposals for consideration.

8. Official Duties: The Nutrition program director has continued to serve as Secretary of the Malnutrition Panel of the US-Japan CMSP, as a member of the Food and Nutrition Advisory Council of the Nutrition Foundation, and as a member of the Scientific Advisory Committee and of the Corporation of the Muscular Dystrophy Associations of America.

The Arthritis Program supports research on many closely related subjects such as connective tissue (collagen) diseases, joints and their contents, inflammation, immunology (autoimmune conditions), chemotherapy of arthritis and connective tissue relating to skeletal muscle.

The three major forms of arthritis are (1) rheumatoid arthritis, the most crippling form, (2) osteoarthritis, the form associated with age and degeneration of joints, and (3) gout, the form which predominates in males and affects joints, especially those of the feet.

Although rheumatic diseases were mentioned in the literature of ancient times, their etiology is still unknown. Strong evidence points to virus or virus-like particles as a possible cause.

The discovery of the HLA W27 histocompatible antigen in patients with ankylosing spondylitis is the most exciting finding of the past few years. It suggests a possible linkage with immune response genes. This antigen is also present in other rheumatic diseases with spine involvement such as Reiter's disease, psoriatic arthritis, juvenile rheumatoid arthritis, and systemic lupus erythematosus. Exploration of this field is being carried out by many investigators.

Osteoarthritis. Metabolic differences have been detected between normal human articular cartilage and osteoarthritic cartilage. The latter can synthesize a_2 chains (characteristic of skin and bone collagen) and B components in contrast to normal cartilage which synthesizes only a_1. This difference in structure may account for some of the functional defects in aged cartilage.

It has also been shown that in osteoarthritic cartilage there are 2 - 4 times the amount and activity of glycosamine glycans over those of normal cartilage.

Immunology. In studying the effect of sodium thiomalate, it was shown for the first time that the gold salt inactivates the functional hemolytic activity of complement, one of the reactants in the pathogenesis and/or perpetuation of rheumatoid arthritis.

It has been suggested that circulating immune complexes of IgG and IgS anti-IgG may have a pathogenetic role in the vasculitis of rheumatoid arthritis. Future studies will attempt to define the cryoprecipitates especially in relation to ability to fix complement.

It has been demonstrated that antibodies to denatured collagen are present in sera of rheumatoid arthritis patients. The occurrence of anticollagen, primarily of the Ig class, is independent of the occurrence of rheumatoid factor.

Using rabbits as experimental models, data suggest that the intra-articular antigen serves as direct stimulus for local synthesis of antibody in the synovial tissue and that it engages in the formation of complement-

binding antigen-antibody complexes that may play a major part in the production and maintenance of synovial inflammation.

Collagen (Connective Tissue). Since collagen is the most prevalent protein in the body (1/3 of body protein), a clear understanding of collagen biosynthesis and control is necessary to understand normal growth and development. Rheumatoid synovial cells contain an "endogenous activator" which induces hypermetabolism. This connective tissue-activating peptide (CTAP) apparently regulates the progression of the inflammatory process from the early exudative phase to the proliferative or reparative stage.

The properties and the formation of the triple helical structure of collagen are being explored by many researchers. Collagen, containing analogs of proline and lysine, has less thermostability than normal, while collagen from ascorbic acid deficient cells is abnormal in configuration and cannot form normal fibers.

Other collagen research has divulged that basement membrane collagen has a higher melting point than skin collagen. This is consistent with the observation that basement membrane collagen contains more hydroxyproline.

Synovial Membranes and Fluid. The trace metal composition of blood serum and synovial fluid of patients with rheumatoid arthritis is being studied. Ligands of trace metals are found in the rheumatoid tissue.

Electron micrographs of synovial membrane cells from patients with multicentric reticulo-histiocytosis reveal for the first time that the Golgi body and smooth endoplasmic reticulum are associated with the development of a substance which accumulates in these cells and produces giant cells. This could aid exploration of the destructive arthopathy in this disease.

Another grantee discovered a predominance of lymphocytes (lymphokines) in the synovial fluid in early rheumatoid arthritis.

Systemic Lupus Erythematosus. It has been shown that in patients with drug-induced SLE syndrome, there is a diminished rate of acetylation of hydralazine. It is postulated that this may be a factor in the susceptibility of patients. Another grantee has postulated that the primary defect in SLE may be a decrease in the T cell function which through decreased T cell suppressor activity allows the formation of autoantibodies.

Gout. Studies on gout are needed which more clearly define the underlying cause of the disease. Studies of interest which warrant additional research involve the manner by which inflammation is produced. Fortunately, drugs such as allopurinol, colchicine, etc., are available to alleviate the symptoms of the disease. A number of leads such as the activation of kallikreins by the Hageman factor and lysosomal products of polymorphonuclear granulocytes, and complement causing the inflammation process are being explored in order to elucidate the cause of gout.

Heritable Disease and Miscellaneous. A grantee has reported that a dramatic corrective effect occurs in Hurler's cells when treated with testicular hyaluronidase. Work in this area is continuing.

Another grantee has postulated that Hurler's syndrome is due to a deficiency of L idurono-sulfate sulfatase.

The discovery was made of a vitamin E-sensitive N-acyl-methionine amido hydralase in vitamin E dystrophic muscle. This represents an interesting effect of a vitamin on an enzyme system.

Workshops. In September 1973 the Arthritis Foundation and the NIAMDD sponsored a workshop on Acupuncture in Rheumatic Diseases which was attended by researchers in arthritis and orthopedics, as well as experts in acupuncture. The summary of this workshop will be published in the Journal of Arthritis and Rheumatism this spring.

In conjunction with the Arthritis Foundation and the American Academy of Orthopedic Surgeons, a workshop was held in Chicago in early April 1974 to determine the function of an "ideal" arthritis center. Results of this workshop will help the Institute in planning future activities.

The NIAMDD, together with the Scoliosis Association of the Academy of Orthopedists, is planning to hold a workshop on scoliosis in November 1974. Preliminary plans for this meeting are already under way.

Training Grants. There are now, in FY 74, 23 active training grants in arthritis.

The area of research covered by the Orthopedics Program includes both basic and chemical studies of bones in the normal and diseased states.

Fractures and Healing. Research on enhancement of bone healing through the application of electrical currents by implanting various devices is being carried on in numerous laboratories. A recent observation from these studies is that Schwann cells may be responsible for the trophic effect of nerves in regeneration, fracture healing, etc. Also, conversion of bone marrow elements to fracture blastoma with subsequent growth and differentiation into cartilage and then bone was impeded by denervation.

Artificial Joints. In the area of artificial joints, the hip replacement prosthesis is most advanced while those for knee, elbow, and shoulder joints are in the embryonic stage. One obstacle in developing artificial joints is obtaining materials which when implanted will be non-toxic, non-antigenic, and show minimal wear and corrosion and thus not necessitate replacement. Another difficulty is keeping the implant immobile so as to develop a long and lasting union between joint and prosthesis. Materials being tested are graphite composition materials and ceramic and plastic-coated metals which include stainless steel, titanium, and a titanium-aluminum-vanadium alloy. This latter material is indeed promising.

Scoliosis. A multidisciplinary approach to scoliosis research is being carried on by two grantees. This includes two-plane radiograph evaluation of the spine, testing of a telemetered Harrington Rod, oxygen consumption studies on patients with the Milwaukee brace and telemetered Harrington Rod, effect of exercise, spinal cord monitoring during scoliosis surgery, psychosocial evaluation, dental problems, pulmonary function, coordination and balance testing, echo encephalographic studies and visual motor perception studies.

Endocrinology. Studies on the sulfation factor and its interrelationship with hormones show that the liver is capable of converting growth hormone to its active agent, somatomedin. A factor secreted by lymphocytes appears to have resorbing activity and is being studied with vitamin D metabolites and prostaglandins for its interaction with bone.

Bone Metabolism. The discovery was made that in studies of newly calcified tissues the destruction of pyrophosphate is necessary for normal mineral formation. Apparently this allows apatite crystal growth rather than permitting conversion of amorphous calcium phosphate to apatite.

Additional findings were made in the phospholipid research area. In the conversion of calcifying cartilage to bone, substantial levels of phospholipases are present in the epiphyseal cartilage, the highest level being in the zone of early hypertrophy where the formation of cytoplasmic processes and budding off of matrix vesicles is very active.

Research on the conversion of serine into cartilage phospholipids (phosphatidyl serine and sphingomyelin) shows that the former lipid is being more rapidly metabolized in calcifying cartilage while the latter lipid is more active in proliferating cartilage.

1,25 dihydroxy cholecalciferol (1,25-DHCC), the active form of vitamin D, is highly effective in eliciting normal bone development and preventing rickets and is potentially useful in treating humans with hypocalcemia and defects of 1,25-DHCC production. However, 1∝ hydroxy cholecalciferol, a synthetic analog of 1,25-DHCC, is also successful in mediating normal bone calcification. Since this synthetic analog is considerably cheaper than 1,25-DHCC, it may be the choice for human use.

Calcium metabolism in the intestines is decreased when bone mineralization is inhibited by ethane-1-hydroxy 1,1 diphosphonate (EHDP). Studies suggest that EHDP inhibits 1,25-DHCC production.

A grantee is perfecting a competitive protein binding assay for 1,25-DHCC, which is the first diagnostic test for this sterol in humans.

Joint Structure and Function. In studies with rabbits orthographic measurements show a significant contracture after extended immobilization. The connective tissue surrounding the immobilized joint showed a significant decrease in glycoamino glycans but not in total collagen. Another grantee discovered that the cartilage lubricating moiety in synovial fluid is a glycopeptide, hexosamine, and also that estrogen reduces the joint stiffness. This latter discovery is being explored further.

Training Grants. The total number of training grants in orthopedics this fiscal year is 12.

In F.Y. 1974 the Dermatology Program Area initiated the contract mechanism necessary to establish a Psoriasis Chemotherapy Screening Program. It is anticipated that a contract will be awarded in early F.Y. 1975. In March 1974, the Second NIAMDD Psoriasis Workshop in conjunction with the National Psoriasis Foundation was held in Bethesda, Maryland. Twenty participants, the majority of them grantees of this Institute, reviewed psoriasis research data that has become available since the first workshop held in October 1971. The apparent defect in cyclic AMP enzyme regulation reported previously is still a valid and primary consideration and being actively pursued by our grantees. Recently described studies have indicated the ability of the cell-mediated immune system to induce hyperkinetic change in the human epidermal keratinocyte. In view of this new information and the long-standing observation that psoriasis is frequently initiated or exacerbated by beta hemolytic streptococcal infections, participants concluded that immunology in psoriasis is an important untapped area for study. One particular area that should be pursued related to the recent reports that patients with specific increased incidence of certain HL-A types (human leucocyte antigen) have unique reaction patterns to certain antigens and manifest a tendency to develop certain diseases. It has now been shown that psoriatics have an increase of two particular HL-A types: a nine-fold increase in HL-A 13 and a 4-5-fold increase in W 17. Epidemiologic data indicate that psoriasis has a complex multifactorial inheritance pattern. Characterization of the immunogenetic factor will mark a major advance in basic investigation of psoriasis. Institute staff is working closely with grantees to plan for designated psoriatic research centers whereby the clinical testing of new anti-psoriatic agents may be carried out in conjunction with the basic research and under carefully controlled conditions and appropriate safeguards.

During the past year the Dermatology Program Director and the Chief of the Allergy and Immunology Branch, Extramural Programs, NIAID have conducted joint investigations and discussions regarding overlapping interests in the disciplines of allergy, immunology and dermatology. It has become increasingly clear that these research and clinically-related areas in general and the programs of the respective Institutes in particular would benefit by some cooperative endeavor. A workshop for the exchange of information and needed interaction between these disciplines would be an appropriate objective of the two Institutes. In cooperation with grantees of both Institutes tentative plans have been made to hold a workshop in immunology and dermatology in the fall of 1975. The topics to be covered will include: IgE-related cutaneous problems including atopic dermatitis and urticaria; immune complexes and complement in cutaneous diseases including vasculitis and bullous disorders; cellular mechanisms and delayed hypersensitivity including cutaneous infections and immunodeficient states.

Significant advances have been made by grantees in the following areas: ultrastructure of sebaceous glands and acne lesions: It has been found that differentiating sebaceous cells in acne do not differ appreciatively from their counterparts in normal glands; however, new ultrastructural studies indicate that the basal lamina around sebaceous glands in some acne lesions deviate from the normal in being either broken or discontinuous or thickened.

It appears that acne involves sebaceous glands that react more like basal lamina of the epidermis than undifferentiated sebaceous cells. Other grantees studying acne have determined the transit time of sebum which is approximately eight days. This will be useful in furthering studies of sebaceous cells and acne.

One grantee whose clinical studies have allowed children with Erythropoietic Protoporphyria to now lead normal lives has confirmed further that the administration of beta carotene is photoprotective and thus far does not seem to have any cummulative toxicity.

Investigators in many fields for years have avoided working with skin because it is so difficult to grow and maintain as well as to differentiate between cell types. One grantee has now been able to isolate skin endothelial cells from blood vessels of a young rabbit ear. These cells can be inoculated into a gel and they multiply into structures resembling native blood vessels. More importantly they may be cultured in the presence of epithelial cells and fibroblasts. These studies represent the final stage in the development of a method to isolate and maintain in cell culture the three major cell types of normal skin.

A goal of the dermatology program area is to obtain a detailed understanding of the mode of action of certain human and animal collagenases and mechanisms involved for controlling their biosynthesis and subsequent activity. This will enable further insight into wound healing and the pathogenesis of certain skin diseases and rheumatoid arthritis.

Dystrophic Epidermolysis Bullosa is a serious blistering disease of the skin. Our grantee has discovered increased collagenase production in this disease. If collagenase is found to play a primary role in the pathogenesis of blister formation, a more specific therapy for bullous disorders could become possible.

The investigations supported in this program are characterized by their wide scope and diversity. They include the application of newly developed physicochemical methods to the study of living systems and molecules of interest, and syntheses of these molecules and their analogs. During the past year substantial results have been reported in these studies, which in the main correlate biological and physical structure of proteins, lipids, carbohydrates, enzymes, etc. with their function in living forms.

Recently reported studies by a supported investigator have thrown new light on basic aspects of muscular activity. Myosin control is one of the major regulatory systems of many muscles. The mechanism of this regulation and the distribution of actin and myosin controls in the animal kingdom is of considerable physiological and evolutionary significance. The analysis of the components of regulation and the understanding of the mechanism of their action is a prerequisite in studying diseased states where regulation may be affected. During the past year these studies have concentrated on the mechanism of myosin-linked regulation, particularly in scallop muscles, and surveyed the distribution of myosin-linked and actin-linked regulations within the animal kingdom.

Myosin-linked regulation requires the presence of a particular light chain which acts as a regulatory subunit of myosin and, in the absence of calcium, blocks sites on myosin involved in the combination with actin. The mechanism of actin-linked regulation is different. Here the regulatory proteins, tropomyosin and actin, block sites on actin and prevent crosslink formation in the absence of calcium. In both actin and myosin-linked regulations, calcium triggers contraction by removing the inhibition.

Significant advances have been made in another area affecting many structures in the body, namely, the basement membranes. Basement membranes are prevalent extracellular matrices throughout the body. The entire vascular endothelium and all epithelial linings rest upon them. Basement membranes are associated with many specialized structures that usually are affected by disease, such as the kidney glomerulus, the lens capsule of the eye, the choroid plexus of the brain and the embryonic membranes which enclose the fetus. Collagen is a major constituent of all basement membranes. A clear, detailed understanding of collagen biosynthesis and control of this synthesis is fundamental to our understanding of normal and abnormal growth and development. Because of its widespread distribution and function, collagen is involved either directly or indirectly in many human diseases, ranging from relatively innocuous conditions such as scar formation to fatal diseases such as generalized scleroderma and the nephrotic syndrome. Basic understanding of normal collagen metabolism whether in skin or basement membrane must precede a rational approach to these diseases. Preliminary studies have demonstrated the occurrence of collagen crosslinks in basement membranes. Basement membrane preparations from anterior lens capsule, Descemet's membrane, and renal glomerulus were reduced with NaB^3H_4 in order to label carbonyl-derived crosslinks. Quantitative incorporation of tritium into the basement membranes was found, similar to the levels observed in fibrous collagens. A considerable proportion of the label was chromatographically identical with

the reduced aldehydes and crosslinks of collagen, suggesting that it is the collagenous portions of basement membrane which contain these compounds. This was substantiated by showing that the isolated collagenous proteins contained substantial amounts of reduced aldehydes and crosslinks. These data further suggest that collagen in basement membranes exists in a precursor form analogous to procollagen.

Improvements in technology have led to increased use of nuclear magnetic resonance as a tool in biomedical resource. One project supported in this program involves studies of properties and reactions of phosphorus compounds in biological systems through use of ^{31}P NMR. Research to date on this aspect of the project has led to the development of methodology whereby ^{31}P NMR is used to detect and characterize members of various classes of phosphorus compounds in intact complex biological preparations. It is anticipated that continued research will bring about refinements and extensions of this methodology so that it can be used with a variety of biological systems at considerably lower phosphorus concentrations than are now required. It appears that the methodology will be of value in practical problems such as the control of environmental phosphorus where it could be utilized for the determination of the types and amounts of various phosphorus compounds in sewage, industrial wastes, detergents, bottom sludges, bacteria employed for phosphorus removal from sewage, etc. It is possible that it may be of use for the clinical diagnosis of diseases which are reflected by an abnormal distribution of phosphorus components of body fluids such as bile, synovial fluid, blood serum, etc.

The specific research to date on biological polyphosphate has provided methods whereby this material can be directly detected and its biological interactions can be followed. This may ultimately lead to an understanding of its function in systems such as the following: microbial systems where polyphosphate has been implicated in energy storage and energy metabolism; mammalian brain and nerve in which polyphosphate has been recently reported to occur; systems involving biological mineralization processes wherein condensed phosphates may conceivably play an important role, etc.

The specific work to date on biological phosphonate and orthophosphate has similar implications which are emphasized by the recently reported discovery of phosphonate in human brain. Recent experiments from supported laboratories suggest that methylenediphosphonate-type compounds may occur in rat brain. Such compounds are known to have strong effects on mineralization processes. The possibility of their natural occurrence suggests that they could conceivably function in the control of biological mineralization and could play an important role in diseases involving this process.

This report constitutes a final accounting for this program. For program reasons it will be administratively terminated as of June 30, 1974, with distribution of the grants presently supported among the other NIAMDD extramural programs.

03 DIABETES PROGRAM AREA

The Diabetes program area supported about 120 research grants in fiscal year 1974. This included basic studies in animals and clinical research in man, and is a slight increase over the number of grants supported in the previous fiscal year. The direct cost dollar value for research grants was approximately $4,946,000 with an additional $836,000 for center grant support. In addition, the program supported 10 training grants and 19 fellowship and career development awards.

In response to Public and Congressional interest, the Diabetes Program has established a series of Diabetes and Endocrinology Centers. Because of tight budgets, last year saw the partial funding of one of these centers. This year, however, the situation improved enough so that full funding was made to the one center, and three additional centers were established. Centers are now starting at The University of Virginia at Charlottesville, The University of Chicago, and The University of Washington in Seattle. The previously established Center is at Vanderbilt University in Nashville, Tenn. The initial emphasis of these Centers is in Diabetes only, and the centers are established "to facilitate, improve coordination or otherwise enhance the productivity of the research environment of the recipient institution." It is hoped that as a result of these centers, basic research findings will receive more immediate application to the patient and his illness.

Another large scale project, now in its 14th year is the series of 13 grants known as The University Group Diabetes Program (UGDP). This group originally compared oral hypoglycemic agents with insulin treatment and diet control in the treatment of diabetes. More recently the oral agents have been discontinued and studies using two types of insulin treatment and/or diet have been made. Although there is still a population under study at the various centers, this phase of the project is nearing completion, and final data will be compiled, analyzed and reported on in the next two years. A long term study of this type is relatively unique, and therefore, the data are of interest to the scientific community.

Aside from the Centers and Special UGDP Studies, the program continues to support individual projects. A major area of support is on studies concerned with the metabolic properties and action of insulin. Approximately 1/3 of the studies may be included in this category. Much of this work is basic in approach - e.g., studies on the sites and methods of insulin action. Another large area of interest concerns the body's response to the products of metabolism, which may be altered in diabetes. Also of interest, are pathological changes seen in diabetes - for example, thickening of basement membranes in some cells. There are changes seen too in small blood vessels and capillaries which cause secondary effects on various organs of the body - e.g., kidney, retina of the eye, brain, heart, etc.

Finally, studies are on-going with experimental diabetes, since the disease is now known to be more than just a lack of the hormone insulin. Animal models which mimic the human form of the disease with its various pathological effects are important in the overall understanding and eventual control

of diabetes as a disease.

The diabetes program director, Dr. LeMar Remmert, died March 13, 1974. Dr. Remmert had been program director since 1968. Much of the program as supported by the NIAMDD was the product of his hard work. He was well-liked by the grantees across the country and his associates at NIH. He will be missed.

The Endocrinology Program area supports basic and clinical research in the areas of structure and function of the endocrine glands, the hormones secreted by the glands, other factors with hormone-like activity, the mechanism of action of the hormones and the effects of other factors involved in their action. Work is progressing in all areas.

Hormone Action

The means by which steroid hormones exert their effect on tissues is still poorly understood. Detailed understanding of the molecular basis of the primary action of protein hormones, including elucidation of amino acid sequences and conformation, identification and binding sites, interaction with target cell receptors, delineation of active sites or centers, relationships between active site and receptor site, chemical structure and biologic activity, effects of chemical modification on biological and immunochemical activity, structural correlates of protein hormone evaluation, and control mechanisms related to synthesis and secretion of the hormones are all areas which need investigation and are being investigated by grantees in this program.

Adrenal Gland

Hormones from the adrenal cortex have widespread effects on gluconeogenesis, fat metabolism, water metabolism, muscle tone, central nervous system, gastrointestinal tract, bone metabolism and the cardiovascular system. The maintenance of structure, growth and secretory action is regulated by the pituitary hormone ACTH. Most of these areas are being studied at the physiologic, cellular and molecular level. Work is in progress on the site of action of ACTH upon adrenal steroidogenesis. Two theories are being pursued: (1) that a transport protein rapidly synthesized at the translational level and having a short turnover time acts to translocate cholesterol within the mitochondria from protected storage sites to sites of action in the formation of pregnenolone and that this protein arises from phosphorylation of ribosomal proteins by the cyclic AMP dependent protein kinase system which is activated by ACTH; and (2) that various factors which modify mitochondrial permeability, increase steroidogenesis with end-product inhibition of pregnenolone. Studies are also in progress on the action of ACTH in dispersed cells in culture, binding of ACTH to receptor sites in plasma membrane of adrenocortical cell, activation of adenylate cyclase and production of cyclic-AMP in response to ACTH. We are also supporting a project to study the stimulation and feedback control of ACTH by physiological techniques and computer simulation - a new approach for using bioengineering concepts to study endocrine control mechanisms.

The adrenal medulla has been an excellent model for [illegible] and studies of the biosynthesis and secretion of hormones. This [illegible] will continue to provide concepts and techniques applicable to the [illegible] nervous.

Thyroid Gland

Function of the thyroid gland is to secrete such quantities of hormones as are necessary to meet the demands the peripheral tissues impose. One of the major components of the hormones is iodine. Hormones are formed and stored in a matrix, thyroglobulin (TBG), released into the blood and transported to various tissues. In one approach to studying these hormones investigators have grown 25 crystals of thyroid hormones, analogues and precursors and have found 10 of them suitable for preliminary X-ray diffraction analysis. All were found to have 3'-iodine in the distal position. These results indicate that the biological activity data from which the distal conformation was inferred are correct. Work is in progress on the purification of intracellular binding proteins for the thyroid hormones T_4 and T_3 which are believed to play significant roles in modifying cellular actions of thyroid hormones. A new radioimmunoassay has been developed for T_3 using thermal inactivation of TBG. In the treatment of thyroid deficiency a replacement therapy has been proposed which would use only T_4 and would allow a constant conversion of T_4 to T_3.

Thyrocalcitonin (TCT) is a polypeptide hormone from the thyroid which lowers plasma calcium and phosphate. Investigators have shown that oral calcium stimulates the release of gastrin or a related hormone which in turn stimulates TCT secretion. The increase in TCT output protects against hypercalcemia. Workers using a calcitonin radioimmunoassay have been able to detect early medullary carcinoma.

Pituitary Hormones

The pituitary gland is responsible for the secretion of several hormones. Growth hormone (GH) is one of the most important of these hormones. Recently one of our investigators has found evidence that the effect of GH on skeletal muscle and perhaps other tissues might be mediated by a reduction in concentration of cyclic-AMP or some other cyclic nucleotide. A novel and important concept has been proposed by another investigator that the steroid environment and possibly other physiological factors affect pleomorphic changes in pituitary hormones secreted from the gland. Changes may be reflected in alterations in the ratio of biological potency and immunological reactivity of the hormones. He will continue this line of investigation of the pituitary hormones FSH, LH and TSH.

Work on the purification of somatomedin, a growth promoting factor, has led to the theory that somatomedin A is but one member of a group of insulin-like peptides controlling cell growth in a wide variety of tissues - a new class of protein hormones regulating the growth of a wide range of tissues. Work is in progress on the elucidation of the physiologic effect of pituitary gonadotropins FSH and LH upon normal ovarian and testicular function as a basic for the development of newer contraceptive approaches and treatment for infertility.

Parathyroid Gland

Evidence has been produced that the preparathyroid hormone after synthesis in ribosomes is transferred to the endoplasmic reticulum where it is converted to parathyroid hormone (PTH) and thereafter is packaged in secretory granule form. Calcium may regulate the formation of PTH in the gland by controlling the amount of pro PTH which is converted to PTH and that which is degraded into biologically inactive peptides. One of our investigator groups has characterized all their available antisera to bovine and porcine PTH which cross react with human hormone with synthetic PTH 1-34. They have 2 anti-sera which have strong affinities for human PTH 1-84 one recognizes the NH_2-terminal region and the other the COOH-terminal region. The COOH-terminal assay is superior in assessing the degree of chronic hyperparathyroidism whereas the NH_2-terminal assay is much better suited for assessing acute changes in parathyroid hormone secretion.

Neuroendocrine System

One of the fundamental problems in human biology is that of neuroendocrine integration. Its complexities require investigation on a broad comparative basis. A new concept regarding the function of the median eminence of the hypothalamus is evolving. This concerns the role of the median eminence in the transport of substances from the ventricular C S F to hypophysial portal blood. Whether its transport role is more important than it secretory role remains to be seen. The discovery of somatostatin which inhibits the secretion of both GH and TSH has opened up a new area of research.

Correlating structural and conformational information with the evolution of neurohypophysial hormones, and the mode of hormone interaction with various macromolecular receptors, it is now possible to prepare neurohypophysial hormone analogues with inhibitory properties which exhibit one specific activity to an exceptionally high degree.

In March of 1973 a contract initiated by the extramural staff was negotiated with the National Academy of Sciences (NAS) to evaluate the National Pituitary Agency (NPA) as a model for other such programs which might be of value to our scientific endeavor. The final report was due from NAS after one year, however, an extension was requested and granted and we now expect to have the report some time this summer.

In the area of Endocrinology during the past year a Human Growth Hormone Symposium, sponsored by the NPA, was held in Baltimore in October and included many of our grantees as participants. A workshop sponsored by the Endocrinology Study Section on Molecular Processes in Secretory Cells was held in January 1974 and stimulated some lively discussion on the latest hypotheses about hormone action at the molecular level. Both of these meetings were attended by the Extramural Staff of this program.

12 - Kidney Disease and Urology Program Area

The Kidney Disease and Urology Program continues to support a large number of studies examining every aspect of the kidneys and the urinary tract in health and disease. Studies include: kidney structure as correlated with function, control of body sodium and water balance, end-stage chronic kidney disease, urinary tract stones (urolithiasis), urinary tract infections, ureteral and bladder physiology, and the mechanisms for cation, anion, vitamin, amino acid, and water transport. Of the approximate 215 research grants receiving support from this program, project size varies from one receiving as little as $15,000/year to one project that was awarded better than one-half million dollars to support a cadre of investigators in the initial year of a five-year commitment. Most certainly the intent of these investigations is for the eventual control and prevention of the primary kidney diseases.

Kidney Transplantation

Transplant rejection is still the most important factor which prevents kidney transplantation from being used as a routine therapeutic measure to resolve the problem of the patient with end-stage kidney disease. Consequently, several institutes, including NIAMDD, support a number of projects that are focusing primarily on drugs that suppress the body's immunologic mechanisms. In addition, investigations on improved immunologic matching of donors with suitable recipients and modes of organ preservation continue to be additional problems that must be solved in the transplantation field.

Chronic Kidney Failure

A study is underway at Johns Hopkins to explore the therapeutic value of administering keto-analogues of the essential amino acids to patients with chronic renal failure and to clarify the mechanisms by which these compounds act. Goals of the investigations include the exploration of long-term keto-acid therapy in patients with the disease and the development of keto-acids for intravenous use. Thus far, three patients have been maintained as out-patients on keto-acids for periods of several months; the longest being maintained for nine months with about 30 per cent of renal function during this period. The two others in the fifth month of therapy have shown little progression of their chronic renal failure and are leading an almost normal existance despite less than 5 per cent residual filtration rate.

Other more directed research programs along these same lines are being supported in other laboratories by this Institute's Artificial Kidney/Chronic Uremia Contract Program.

Cooperative Studies

Support of two multi-center collaborative studies are underway to assess the balance between the relative therapeutic effectiveness and toxicity of cyclophosphamide and adrenocortical steroids, one in children and the other in adults with the nephrotic syndrome. The basic premise for these studies is that because of the lack of knowledge regarding the etiology of this disease prevention has not been possible and that the attempts toward treatment

have followed two general approaches of trying anti-inflammatory or immunosuppressive methods of treatment. Unfortunately, as yet there is no clear cut evidence that either form of therapy has proven to be effective in treatment of the disease.

Urinary Tract Stones

An area in which a slow but progressive increase of investigative activities has evolved is the study of kidney stone formation. One group of investigators contends that calcium oxalate crystals are critical for the formation of most stones and therefore, the thrust of their investigations is on the growth and structure of these crystals in different media in the presence of various additives, such as sodium pyrophosphate, sodium tripolyphosphate, magnesium nitrate, creatine, and urea. An interesting revelation of these studies indicates that the additives appear to aid the dissolution of calcium oxalate crystals by preventing the conversion of the trihydrate form to the more stable monohydrate form; the former in turn dissolving independently with time.

Organ Culture Studies

Work with embryonic kidney, as reported by one laboratory, includes studies of the effects of varying concentration of atmospheric oxygen and the administration of adrenocorticosteroids. These investigators point out the importance of their studies as being particularly relevant to the problems of congenital anomalies of the kidney, especially paramount in importance are the effects of adrenocorticosteroids upon embryonic and fetal tissues when administered to the pregnant mother.

Electron Probe

The powerful method of electron probe microanalysis adopted for analyzing the ionic composition (sodium, potassium, calcium, magnesium, chloride, phosphorus, and sulfur) of glomerular ultrafiltrate and proximal and distal tubular fluid samples is operational in a few laboratories conducting investigations on the kidney. The advantages of the system are its specificity for many ions which are presently difficult to identify, the minute (picomolar) samples needed and the preservation of the sample following analysis. With this new technique, insight into transport mechanisms can be gained by comparing the tubular cells to the tubular fluid ionic content. It is also anticipated that methods can be developed with this technique to study the handling and location of drugs within the kidney and other organs as well.

International Nephrology Congress

The plans are already underway for the Sixth International Congress of Nephrology to be held in Florence, Italy during the week of June 6-14, 1975. As in the past, the American Society of Nephrology will be submitting a travel grant application requesting funds for support of a United States contingent at the international meeting.

Benign Prostatic Hyperplasia (BPH) Workshop

A two day workshop on Benign Prostatic Hyperplasia, to be held at the NIH on October 3-4, 1974, is being organized and will be supported by this Institute. The meeting will have a multidisciplinary focus, assured by having participants from the disciplines of urology, endocrinology, biochemistry, microbiology, and virology, to meet the intent of outlining an objective evaluation of what is known about the disease and hopefully open new approaches for future investigations. Since BPH is known to occur in 50 per cent of all males over the age of 50 and is known to cause many physical problems including urinary tract infection, renal insufficiency, bladder dysfunction, calculous disease, hypertension, and significant morbidity and mortality due to the only satisfactory treatment, surgery, the workshop is considered very timely.

Investigations into hematologic disorders have been supported by NIAMDD since the beginning of the extramural grants program in the early 1950's. Studies on the metabolism of vitamin B_{12} and its role in pernicious anemia were the subject of the earliest grants. Todays program is considerably more diverse and extensive accounting for about 10% of the Institute's extramural research grant expenditures in FY '74. The following report identifies four major areas of research effort that represent NIAMDD's current responsibility in the field of hematology.

Non-Genetic Anemias and other Disorders of Blood Cell Production

Although the number of projects and the funds granted for these investigations represent a relatively small segment of the total NIAMDD budget in Hematology (about 15% in 1973-1974), this area continues to be a most important part of the total program. This Institute's research grant support accounts for nearly all of NIH's extramural expenditures for such diseases as iron and other nutritional deficiency anemias, aplastic anemia, sideroblastic anemia and anemias secondary to chronic diseases.

Significant progress has been made through grant support in improving our understanding of iron transport by the convincing confirmation of the Fletcher-Huehns hypothesis that the iron transport protein transferrin does indeed deliver iron atoms from each of two iron-binding sites in a selective fashion to receptors on cell surfaces. Long-standing ferrokinetic models will have to be reexamined and revised in light of this work which in turn should help clarify our understanding of iron transport in pathologic states.

Biochemical and physiological abnormalities are now beginning to be recognized in sideroblastic anemia, a disorder for which the underlying cause and consistently effective treatment remain unknown. Two mitochondrial enzymes of heme biosynthesis, the rate limiting enzyme ALA synthetase and ferrochelatase, the last enzyme in the pathway, have been observed to have decreased activity in patients with idiopathic sideroblastic anemia.

Building on the radioassay for serum folate reported last year, a test has been developed for red cell folate levels and folyl polyglutamate levels which should be helpful in rapidly assessing the severity of tissue folate deficiency. The superiority of the new radioassay to the microbiologic assay should aid studies on deranged folate metabolism and its clinical manifestations in uremia, in patients on Dilantin, ethanol or oral contraceptives, in patients with hematologic malignancies or with liver disorders.

Inherited Disorders of Hemoglobin Production

Quantitation of the activities supported in this area continue to show the momentum that was generated by the special legislation for both sickle cell anemia and Cooley's anemia. One-third of the funding in NIAMDD's Hematology program in FY '74 can be identified as related to the hemoglobinopathies.

In an effort to determine whether there has been a selective exclusion from physically demanding occupations of individuals with sickle cell trait (Hemoglobin AS), black players on National Football League teams were screened electrophoretically for their hemoglobin phenotype. The results indicated that the incidence of hemoglobin AS in this group (6.74%) did not differ significantly from that found in the black population tested by the Veterans Administration Cooperative Study (7.74%). The new information thus generated should be helpful in putting this genetic condition in perspective by showing that the presence of hemoglobin AS is not detrimental to athletic capability.

The NIAMDD continued its active role in the Inter-Institute Committee on Cooley's Anemia (IICCA) during the past year. Activities related to Cooley's Anemia are coordinated through this Committee and cooperation with the National Heart and Lung Institute in this area was strengthened through concerted planning. In the second annual report on Cooley's Anemia generated by the IICCA, NIAMDD documented support of over one-half the total research projects on Cooley's Anemia funded by NIH in 1974. While there was a limited number of new programs that received funding during this period, it was notable that the development of new drugs for effective iron chelation began to receive additional attention both through research and contract mechanisms. A surprisingly strong response came from both academia and industry to the RFP issued in October 1973 for the development and testing of new iron chelators for the treatment of iron overload in patients with Cooley's Anemia. Contracts for development and testing of new drugs are expected to be negotiated before the end of FY '74.

Enzymatic and Metabolic Defects of Blood Cells

As in other tissues of the body, the blood cells are dependent on enzymes to extract energy from a substrate, such as glucose, and to utilize it in carrying out their functions. The accessibility of blood cells and the metabolic characteristics they often have in common with other tissues favor their use as model systems as well. Biochemical studies of enzymes together with physiological investigations of the metabolism and transport activities of blood cells constitute an area of research effort that accounts for close to 40% of the entire program's funding.

Important progress has been made in attempts to utilize red blood cells for enzyme replacement therapy. Normal blood cells are stripped of their hemoglobin content and loaded with the enzyme to be replaced. In mice deficient in beta glucuronidase, red cells loaded with the enzyme have been administered resulting in consistent observation that the enzyme has localized in the animal's liver and red blood cells. This experimental therapeutic approach has been initially successful in a patient with alpha galactosidase deficiency.

A systematic assessment of Heinz bodies created in red blood cells by cation movements has shown these cells to develop an increased leakiness to potassium causing them to shrink in sodium media such as plasma. Physiologic

studies such as these have demonstrated the relationship between poor survival of these cells in the circulation and changes in their membrane permeability. Such an *in vivo* demonstration of another volume regulatory mechanism which is not associated with the classical sodium pump is of considerable physiological importance in other cell types as well.

Immunohematology

Under this rather broad heading are included a number of separate areas that are interrelated by virtue of their immunologic considerations. Studies of human complement and genetic variations of its hemolytic activities, investigations of erythrocyte coating substances in autoimmune hemolytic anemias, work in identifying responsible mechanisms in cold agglutinin disease and studies of human lymphocyte transplantation antigens (HL-A) are supported in this segment of the program. Such projects accounted for 15% of the total research grant funding in the Hematology Program Area in FY '74.

The conviction that susceptibility to disease is ultimately genetically determined has led to many studies in the past attempting to associate certain diseases with known genetic markers. Recent findings of high associations of HL-A antigen W27 with ankylosing spondylitis, Reiter's disease, anterior uveitis and juvenile rheumatoid arthritis suggests a common etiology and constitutes an important development in our knowledge of these diseases. Other HL-A antigens have also been recently shown to be significantly associated with such diseases as coeliac disease, dermatitis herpetiformes, myasthenia gravis, psoriasis and multiple sclerosis. These findings indicate that the HL-A antigen system provides us with a genetic marker for "susceptibility" genes for a number of human diseases.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR
EXTRAMURAL PROGRAM ACTIVITIES

ASSOCIATE DIRECTOR'S REPORT

The year past has proven to be an interesting one chiefly in demonstrating how slowly evolution may occur following reorganization. Still no resolution has occurred in the administrative and personnel language although there has been much paper work performed toward these ends. In this period there has been sufficient attrition in positions over the entire extramural program to permit some implementation of the reorganization but this has not taken place.

It is still planned to have the Administrative Officer-EP directly responsible to this Associate Director but this has not been consummated in personnel terms, though it has been operationally. The greatest step ahead was in naming of a Chief in the Data Storage Section to give effective day-by-day leadership there, an impossibility when the Associate Director was "acting." To counter this advance our "operations officer," a long serving employee was retired and her understudy has maintained the precarious balance in this part of the organization. Consideration must be given to filling this post, hopefully by promotion from within, particularly if the load increases in this unit with the reconstitution of the fellowship and training programs.

As this is being written the retirement of the "old-time" Research Grants Officer is nearing reality. This post, as such, was really eliminated in the reorganization and so at this time no replacement is planned. Duties of this individual and responsibilities of the post will be amalgamated into this office.

The general work responsibility continues to be supportive for all the Institute's Extramural Programs. We still provide personnel services to: Extramural Programs as middlemen between the Institute Personnel Office and our operating levels, administration re space allocation, procurement of services and supplies through the Institute Administrative Office; award our grants for the various program areas within the stated "clusters."

As last year we feel very fortunate to have accomplished our ends, sometimes not on the time schedule we had set but eventually. We are aware that there is more to do than we can do with the available personnel. I am amazed that in spite of our frustrating slowness of promotions, and promises unkept we have not failed as yet. Onward!

Rather than attempt a comprehensive review, this report deals with one of the institute's chief aims in the training area, the design of an appropriate institutional component for the new fellowships program. Contributory here have been review of the issues by our Council in a special meeting convened for this purpose last year, and proposal drafted by Dr. David Nathan. This report presupposes familiarity with these. Its substance is a survey of fellowship review findings just emerging for the light these could throw on the institute's planning.

The special circumstances of launching the new program prompt the expectation that new entrants into research training would be under-represented, those from the MD population especially. In a population made up largely of individuals with some research experience behind them it seemed reasonable to expect a fair proportion of those receiving their support through AM training grants. The proportion of these to the total deserves study for indication of the relative contribution of AM support to the total picture, first in terms of numbers of individuals, and second in terms of quality. Sufficient progress has been made in analysis of study section recommendations to give figures on these matters. No attempt is made to subdivide findings by institute program.

Determination of AM institute training relatedness: some applicants had trained with sponsors we support through the training grant, with or without stipend support from the grant; others would use the F-22 for their first contact with AM-training-supported sponsors; a third group exhibits both, having had previous contact and seeking further opportunity through the F-22. For purposes of this report no distinction is made among these.

AM-related group/total: Of 365 applications assigned to AM the results of review for 275 were available for this analysis. Care was taken to include all the recommendations on AM applications from any study section.

	AM -related	Remainder	Total
PhD's	27	125	152
MD's & other professional degrees	53	70	123
Total	80	195	275

Thus almost a third of the applicants either have had or are seeking experience with sponsors supported through our present program. The figures differ between the two degree groups in the manner expected. With completion of reporting it will be useful to distinguish the several institute programs and to separate those seeking their first contact from those seeking continuation.

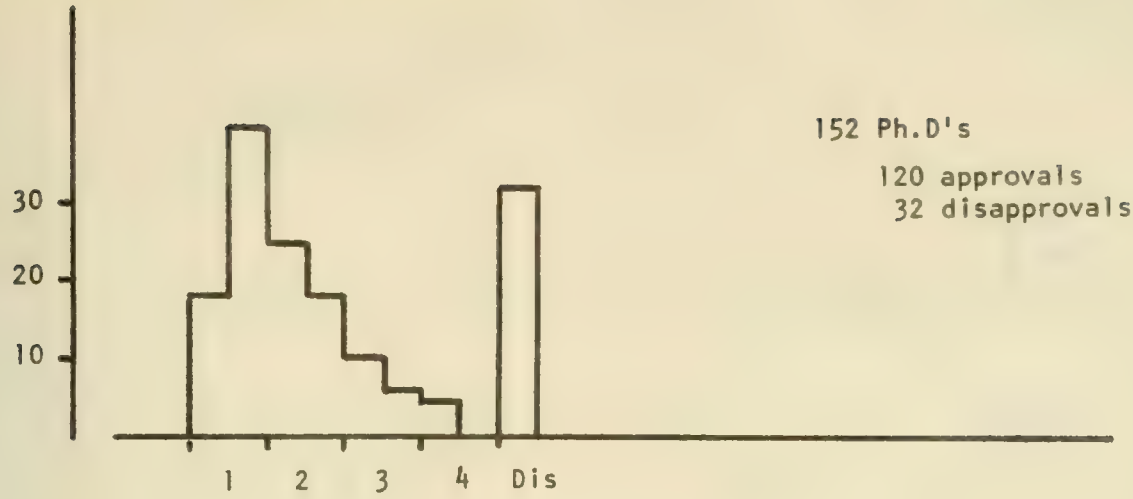
Quality assessment by Study Section: This is shown in the attached frequency distribution diagrams. The priority score assigned by the study section is displayed along the abscissa. For this purpose the 100 - 500 range was divided into 8 equal segments, with a 9th for disapprovals. The numbers of applications falling into each category is shown as the ordinate in units of 5. The first pair of diagrams (table 1) shows the priority distribution for PhD's and MD's. The second set of four (table 2) shows further subdivision to allow comparison between the AM-related group and the remainder within the two degree groups.

In table 1 the distributions exhibit a consistent pattern with a peak to the left of mid-range and skewing toward the poorer priorities. When the two degree groups are further divided to yield comparison of the AM-related group with the remainder, the skewing is found to be more prominent in the latter. With a more precise definition of the AM-related group the data affords a glimpse of the selection that comes about when the program director urges the more promising trainees to seek individual support. When it comes to planning the working relationship between individual and institutional awards, the finding has considerable interest whether it be the result of such deliberate action or of preselection by other mechanisms.

Comparison of quality assessment by Study Section & Training Committee:

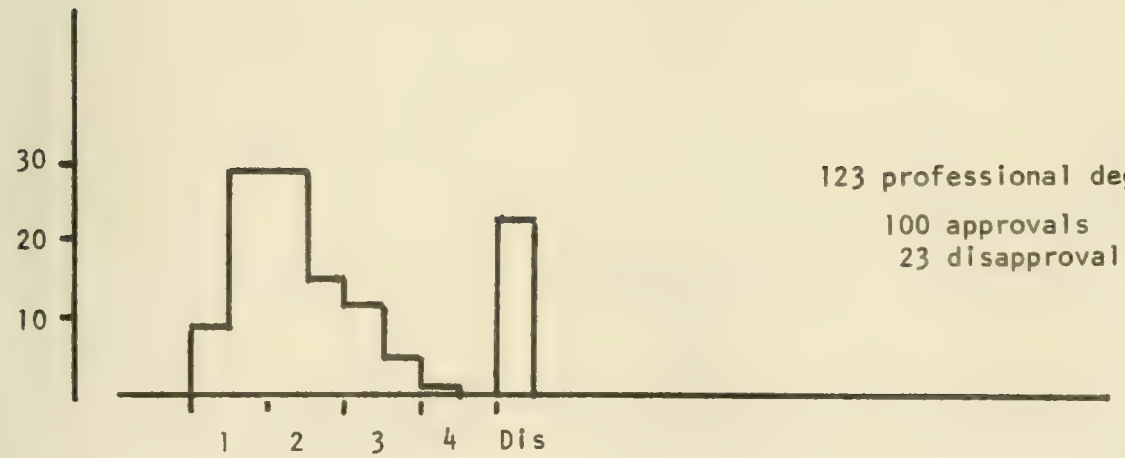
The comparison is one between study section assessment of individuals and training committee assessment of institutional units. Two factors make for a much curtailed priority range in the latter. The training grant approved with a poor priority went unfunded, and more recently our failure to fund even intermediate priorities discouraged their use. Thus it is not surprising that three-quarters of the entries in the table represent training grants with priorities better than 200 (table 3). Of interest is the striking difference in study section priority distribution when comparison is made between training grants with priorities better than 150 with those falling between 150 and 200. The median study section score for the former falls in the 100-150 range, and for the latter in the 200-250 range. The difference is not limited to the higher disapproval rate in the latter.

Finally, the institute will want to examine carefully the significant representation of disapprovals associated with training grants in the 100-200 range. Amounting to 20% of the total, the proportion is no smaller in this highly recommended group than the remainder. Further scrutiny is needed, particularly with reference to the review summary, to discern the kinds of reservations entering into the recommendation. Institutional autonomy in appointments emerges from Dr. Nathan's analysis as the key feature of training grants to be preserved in any plans for institutional-type support. He would be among the first to agree that autonomy should be understood as autonomy-within-limits. Perhaps the study section membership in their capacity as directors of their respective training programs would agree that such limited autonomy should include the right to make appointments of the kind disapproved in individual fellowship review. Further scrutiny of this group should prove illuminating.



152 Ph.D's

120 approvals
32 disapprovals



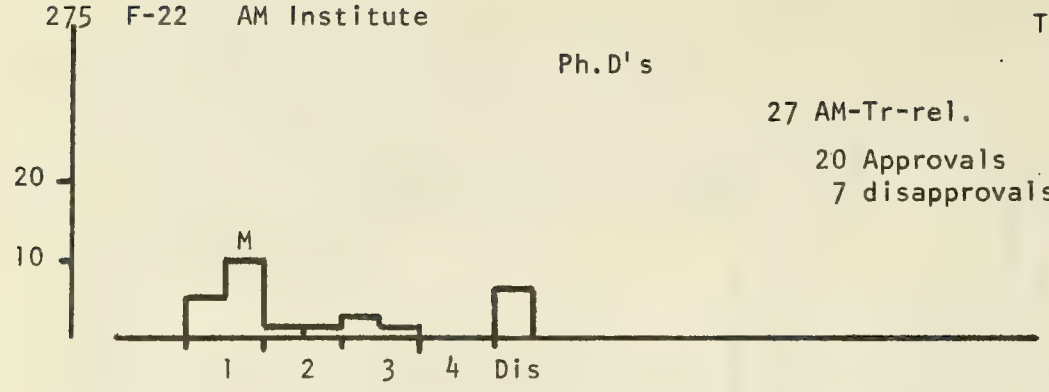
123 professional degrees

100 approvals
23 disapprovals

WB 5/16/74

Table 2

Ph.D's



27 AM-Tr-rel.

20 Approvals
7 disapprovals

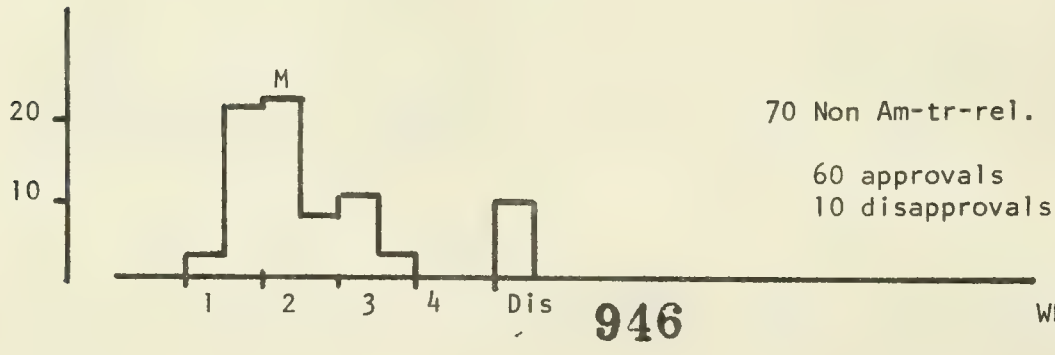
125 Non AM-Tr-rel.

100 approvals
25 disapprovals

Professional Degrees

53 AM-Tr-rel.

40 Approvals
13 disapprovals



70 Non Am-tr-rel.

60 approvals
10 disapprovals

WB 5/16/74

Table 3

STUDY SECTION PRIORITY RANGE

TRAINING COMMITTEE PRIORITY RANGE	-1-	-2-	-3-	-4-	Dis.
-1-	16	14	4	4	1	3
-	3	5	6	4	1	1	9
-2-	1	1	1			1	2
-			1		1		4
-					1		1
-							
-							
-							

ANNUAL REPORT FOR ADMINISTRATIVE SERVICES - NIAMDD-EP

During F.Y. 1974, with the Administrative Office fully staffed and all services operating from a single office for the first time in many months, this Office provided the usual full-range of functions to Extramural Programs.

The personnel function was perhaps the least evident due to the imposed restrictions which resulted in few promotion "points" and even fewer vacant positions.

As anticipated, the travel function was not noticeably affected by the F.Y. 1973 phase-out of training grants and fellowship awards. Only during the latter months of F.Y. 1974, when restrictions on travel were imposed in the interest of energy conservation, was any reduction in staff travel evident and that was minimal.

The creation of the Extramural Program's first conference room was completed during F.Y. 1974. Considerable time and expense went into what has resulted in a most attractive and very functional area which will be of benefit to the entire staff.

The largest single problem area affecting the Grants Management Branch this year was the ordered release of impounded FY 73 funds and the ultimate decisions reached of how to best utilize these funds. These released funds amounted to $13,155,000 for Research Grants, $3,322,000 for Training Grants, and $899,000 for Fellowships. Certain constraints were placed on how we could obligate these funds by the courts, the Department, and, of course, NIH. In research the funds were used by a combination of checking the fiscal 73 competing inventory of applications and paying those deemed eligible as well as transferring certain funded competing 74 awards to 73 impounded funds. This latter group was made up of applications that were actually reviewed during fiscal 73 but funded from 74. The training grant situation proved to be a bit more murky in terms of how to utilize the released funds. Finally, it was decided to supplement the 73 training awards using released impounded funds.

Another budget factor affecting the decision-making process was the "add on" of over $12 million to the research grants budget. This occurred about midway through the fiscal year. This increase coupled with the release of impounded funds caused us to seriously consider what the effect of this would be in fiscal 75 and 76. To keep our commitment level as reasonable in 75 and 76 as possible, it was decided to restore $4 million of "previously reduced" 74 awards. This process involved revising a large number of previously issued 74 awards using the "add on" 74 dollars.

During the course of the year, it was decided that the Diabetes Program Area should have its own allocation of funds in the research grant area. This now makes three program areas that require separate calculations in the fiscal area.

Grants Management Branch participated in many site visits on P-17 applications during the year. These applications were for research in diabetes, peptic ulcer, liver disease, and obesity. Many of these applications involved consortiums. This is a situation where the grantee institution contracts out certain of the research to other institutions (separate entities). Since this is a relatively new and complicated process, GMB accompanied the site visit teams to make sure that the grantees understood our consortium policy.

All these factors (and others) combined to put substantial strain on GMB manpower. Even more than in previous years it was apparent that additional help was needed in the grants management, fiscal, and typing areas of our group.

ANNUAL REPORT OF THE GRANTS OPERATIONS SECTION - NIAMDD-EP

The grants operations section is responsible for processing all research, training, fellowship and career applications, preparing council material, maintaining all grant files and distributing the mail.

The retirement of the section chief, which brought the number of personnel down to seven, and the advent of the new fellowship program have increased the work load of all members of the section.

OFFICE OF THE ASSOCIATE DIRECTOR FOR PROGRAM ANALYSIS
AND SCIENTIFIC COMMUNICATION

SCIENTIFIC COMMUNICATIONS SECTION

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The Scientific Communications Section is charged with the general programming and planning of communication activities concerned with the various scientific areas under the purview of the NIAMDD. The present activities can be roughly divided into four main categories:

1. Current awareness literature services;
2. Retrospective literature studies;
3. Conferences and workshops; and
4. Other aspects of communication, such as the production of motion pictures, thesaurus development, etc.

Current awareness literature services are provided by the Institute in several specialized areas. There are four publications which provide subject organized listings of literature citations and one publication which expands to include abstracts. GASTROENTEROLOGY ABSTRACTS AND CITATIONS, now in its eighth volume, is intended to provide selected abstracts and literature citations in all aspects of gastroenterology, biochemical or clinical. Additionally, complete coverage of four selected gastroenterological areas is being performed and compiled into formatted magnetic tapes, suitable for searching through the facilities at DCRT. This additional information, along with the regular issues of GASTROENTEROLOGY ABSTRACTS AND CITATIONS, are being utilized by the Digestive Diseases and Nutrition Program of the NIAMDD. The ARTIFICIAL KIDNEY BIBLIOGRAPHY (eighth volume), ENDOCRINOLOGY INDEX (seventh volume), DIABETES LITERATURE INDEX (ninth volume), and INDEX OF DERMATOLOGY (sixth volume) all contain citations to current world-wide literature which have been included by the National Library of Medicine in their Medical Literature Analysis and Retrieval System (MEDLARS). In order to serve those who are interested in highly specific areas, we have cooperated with the National Library of Medicine--and in one case also with the University of Minnesota and the University of Rochester--to produce structured, hierarchically-arranged indexes which function as in-depth current-awareness tools for easy review of particular subjects. The standardized terminology used in MEDLARS makes these publications invaluable for retrospective literature searching.

An Editorial Advisory Committee of outstanding scientists working in the area covered by the publication have been assembled for each publication to ensure the continuation of the high quality of these publications.

Expansion of the ARTIFICIAL KIDNEY BIBLIOGRAPHY is underway to include other aspects of relevance to research on artificial kidneys such as kidney diseases, nephrology, and urology. The second experimental issue was printed and a meeting of the Editorial Advisors was held to discuss the experimental issue. Based upon their recommendations, further materials were submitted to them for their comments and suggestions. The second issue was distributed at both the American Society of Nephrology meeting and the Seventh Annual Contractors' Conference. Comments, suggestions, and criticisms have been received from recipients of this second issue. Work with the International Committee for Nomenclature and Nosology of Renal Disease to include the International Committee's nomenclature in the bibliography is progressing. Work with the National Library of Medicine's Medical Subject Heading Section is on-going to include kidney-related vocabulary in the National Library of Medicine's computerized vocabulary.

Clearance from the Office of Management and Budget to publish the new Kidney Disease and Nephrology Index as a recurring bibliography which was requested last year is still pending.

Conferences and workshops which are sponsored by NIAMDD are planned and organized by the Scientific Communications Section. In addition to the organization, which usually includes physical arrangements as well as program planning with the conference chairman, the publication of the proceedings also falls under the auspices of the Scientific Communications Section. This year eight conferences and workshops, sponsored either completely or jointly, were the Seventh Annual Contractors' Conference, Acute Renal Failure Conference, Workshop on Acupuncture in Rheumatic Diseases, Human Growth Hormone Symposium, Conference on End-Stage Diabetic Nephropathy, Second NIAMDD Psoriasis Workshop, Workshop on Arthritis Centers, and the Artificial Kidney-Chronic Uremia Program Adequacy of Dialysis III.

In addition, the contract for the Assessment of the Clinical Trials of Oral Antidiabetic Agents has continued through this past year. A report will be forthcoming before the end of the fiscal year.

In cooperation with the National Cystic Fibrosis Research Foundation, a comprehensive bibliography is being compiled and indexed. Its arrangement is such that it will be able to be updated from year to year.

NIAMDD staff have access to MEDLINE, National Library of Medicine's on-line MEDLARS (Medical Literature Analysis and Retrieval System), through NIAMDD's call number. The Scientific Communications Office coordinates this use and acts as a reference source for the NIAMDD staff.

In addition, numerous other activities which bear on scientist-to-scientist communication as it affects the Institute are dealt with frequently. Areas where further emphasis should possibly be placed are continually in review.

Project Title: Artificial Kidney Bibliography

Professional Personnel: Mrs. Billie B. Mackey
Dr. Keatha K. Krueger
Dr. N. B. Cummings

Man Years:

Total:	0.9
Professional:	0.6
Other:	0.3

Project Description:

The Artificial Kidney Bibliography, now in its eighth volume, is a quarterly recurring bibliography on kidney failure, artificial kidneys and kidney transplantation, which is being produced with the aid of the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS) by the Scientific Communications Section, NIAMDD. It contains about 500 citations per issue with the citations arranged hierarchically to enable similar subjects to appear together, and with an author index included in each issue. A current listing of technical reports (both annual and final) submitted to the Artificial Kidney - Chronic Uremia contract program is included in each issue. These reports are available through the National Technical Information Service for a nominal fee.

The Artificial Kidney Bibliography is made available without cost to National Institutes of Health grantees or contractors who are involved with work related to artificial kidneys, as well as to other government agencies with responsibilities in the area, medical school and hospital libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office, at a nominal price. The mailing list was circularized to insure currency.

An expansion of this bibliography is underway with plans to include other aspects of relevance to research on artificial kidneys, such as kidney diseases, nephrology, and urology. An Editorial Advisors' meeting was held to discuss the first Experimental Issue, and further MEDLARS searches were designed based upon the recommendations of the Editorial Advisors. A second Experimental Issue was printed and the Editorial Advisors convened to discuss the changes incorporated in the second issue. Based upon their recommendations, further materials were submitted to them for their comments and suggestions. The second issue was distributed at both the American Society of Nephrology meeting and the Seventh Contractors' Conference. Comments, suggestions, and criticisms have been received from recipients of this Second Issue. Work with the International Committee for Nomenclature and Nosology of Renal Disease to include the International Committee's nomenclature in the bibliography is progressing. Work with the National Library of Medicine's Medical Subject Headings Section is on-going to include kidney-related vocabulary in the National Library of Medicine's computerized vocabulary.

Clearance from the Office of Management and Budget to publish the new Kidney Disease and Nephrology Index as a recurring bibliography which was requested last year is still pending.

Project Title:	Diabetes Literature Index
Contract Number:	PH43-67-663
Amount:	$85,239
Contractor:	University of Minnesota
Project Officer:	Dr. Keatha K. Krueger
Other Professional Personnel:	Mrs. Billie Mackey
Man Years:	Total: .52 Professional: .32 Other: .20

Project Description:

Diabetes Literature Index, a monthly current-awareness publication of the NIAMDD (now in its ninth volume) was brought about through the cooperative efforts of NIAMDD, NLM, the American Diabetes Association, and three universities (University of Minnesota, University of Rochester, and Case Western Reserve University), as well as a number of individual scientists.

Each month MEDLARS magnetic tapes, which contain all of the current biomedical literature citations used for the preparation of the NLM's Index Medicus, are used for identification of all diabetes-related bibliographic citations. Through 1970 the Diabetes Literature Index has consisted of a keyword in title index and an author index resulting from a computer printout. Starting in 1971, the magnetic tape version of these indexes (prepared at the University of Minnesota) is used to generate copy for offset printing by the Government Printing Office on their Linotron. There are now three formats consisting of a hierarchical subject index of core diabetes literature, an abbreviated keyword in title index of all diabetes-related literature, and an author index including index terms along with the citations (for the first author only). Printing the citations under a preconceived hierarchical structure, which groups related subjects, makes it easier for the user to scan his particular interests in one section.

Diabetes Literature Index is published monthly by the Office of Scientific Communication, NIAMDD, and is available, free upon request, to qualified individuals with NIH grants or contracts who are working in the field of diabetes research, as well as to other government agencies with responsibilities in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, Government Printing Office.

Project Title: Endocrinology Index

Professional Personnel: Mrs. Billie B. Mackey
Dr. Keatha K. Krueger

Man Years:

Total:	.7
Professional:	.4
Others:	.3

Project Description:

The Endocrinology Index, now in its seventh volume, is a bimonthly publication of the NIAMDD, which is produced by the National Library of Medicine's Medical Literature Analysis and Retrieval System (MEDLARS). Each issue contains about 4,000 citations of the current world literature in endocrinology. The cications are grouped in eight categories (pituitary, thyroid, etc.) and each category has a preconceived hierarchical organization of subject headings. A list of the index terms assigned to the original article by the indexer appears in the author section. Author and subject indexes appear in each issue. Work was initiated with the Medical Subject Headings Section of the National Library of Medicine for the inclusion of endocrine-related vocabulary.

Work with the Medical Subject Headings Section of the National Library of Medicine for the inclusion of endocrine-related vocabulary in their computerized vocabulary is on-going. Selections in the area of neuroendocrinology from the five publications of the Brain Information Service and books from the National Library of Medicine's Current Catalog were included periodically.

The Endocrinology Index is available without cost to NIH grantees and contractors with interest in the field of endocrinology, and to medical libraries. It is also for sale at the Superintendent of Documents, U.S. Government Printing Office at a nominal price. The mailing list was circularized to insure currency.

Project Title:	Gastroenterology Abstracts and Citations
Contract Number:	NIH 73-2227
Amount:	$90,019
Contractor:	Franklin Institute
Project Officer:	Dr. Keatha K. Krueger
Other Professional Personnel:	Dr. James Burks
Man Years:	Total: .25 Professional: .1 Other: .15

Project Description:

In January 1966 the NIAMDD began publication of a current-awareness journal for gastroenterologists. Gastroenterology Abstracts and Citations, now in its eighth volume, appears monthly. Each issue contains approximately 350 abstracts of the most significant gastroenterological literature published in the world and approximately 750 citations covering other relevant gastroenterological literature. The abstracts and citations are divided into three main categories--Pre-clinical Sciences, Diagnostic Procedures, and Gastrointestinal Diseases. These categories are subdivided into more specific groupings. Each issue contains a subject and author index and these are cumulated annually.

Since 1973, in addition, the contractor is furnishing complete literature coverage on four selected areas, formatted on tape suitable for searching, for the benefit of the Digestive Diseases and Nutrition Program of the NIAMDD.

Gastroenterology Abstracts and Citations is available, free upon request, to qualified investigators with NIH grants or contracts in the field of gastroenterology, as well as to other government agencies with responsibilites in this area, medical school and hospital libraries. In addition to the free distribution of this publication, it is for sale at a nominal price by the Superintendent of Documents, U.S. Government Printing Office.

Project Title: Index of Dermatology

Professional Personnel: Mrs. Billie B. Mackey
Dr. Keatha K. Krueger

Man Years:

Total:	0.3
Professional:	0.2
Other:	0.1

Project Description:

To further the NIAMDD Dermatology Program as well as respond to the increased interest and activity in dermatology, NIAMDD undertook the publishing of Index of Dermatology in April 1972.

The Index of Dermatology is a monthly recurring bibliography on skin diseases, both general and specific, with aspects such as immunology, mycology, virology, and oral disease. The Index which is being produced monthly with the aid of the National Library of Medicine's MEDLARS (Medical Literature Analysis and Retrieval System) by the Scientific Communications Office, NIAMDD contains about 1,200 citations per issue. The citations are arranged hierarchically to enable similar subjects to appear together, and an author index is included in each issue.

Fourteen outstanding scientists working in the area of dermatology have been assembled as Editorial Advisers for continuing the high quality of this publication.

The Index of Dermatology is made available without cost to National Institutes of Health grantees or contractors, who are involved in work related to dermatology, as well as to other government agencies with responsibilities in the area, medical school and hospital libraries. In addition to the free distribution, it is for sale by the Superintendent of Documents, Government Printing Office, at a nominal fee. The mailing list was circularized to insure currency.

Project Title: Artificial Kidney - Chronic Uremia Program
Sixth Annual Contractors' Conference
Proceedings

Professional Personnel: Dr. Keatha K. Krueger

Man Years:

Total:	0.1
Professional:	0
Others:	0.1

Project Description:

The Sixth Annual Contractors' Conference, held February 12-14, 1973, brought together about 160 contractors' key staff members with consultants to the Program and Institute staff. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on program progress and projected plans for the future.

The conferees, in both plenary sessions and intensive individual workshops, communicated research results in the main areas of the Artificial Kidney Program: 1) Biochemistry and Pathophysiology; 2) Membranes and Other Materials; 3) Nonthrombogenic Surfaces; and 4) Cannulae. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of 68 currently active research and development projects, the Program's consultants and its staff. It is anticipated that this and future conferences will significantly assist attainment of the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The Proceedings of the Conference (DHEW Publication No. (NIH) 74-248) were compiled and given wide distribution to the community of scientists interested in the field of artificial kidney and chronic uremia.

Project Title: Artificial Kidney - Chronic Uremia Program Seventh Annual Contractors' Conference and Proceedings

Professional Personnel:
Dr. Robert J. Wineman
Dr. Fernando Villarroel
Dr. Keatha K. Krueger
Mrs. Billie B. Mackey

Man Years:

Total:	0.4
Professional:	0.2
Others:	0.2

Project Description:

The Seventh Annual Contractors' Conference was held January 28-30, 1974. About 175 participants who were contractors and their staff, consultants to the Artificial Kidney - Chronic Uremia Program, and Institute staff attended. The three-day session involved extensive study of existing contract-funded research and development projects with emphasis on intercommunication of program progress and projected plans for the future. The presentations were grouped in three sessions: 1) Biochemistry and Pathophysiology; 2) Membranes, Cannulae, and Nonthrombogenic Surfaces; and 3) Device Development, Therapy and Its Evaluation. The conferees in both plenary sessions and intensive individual workshops, communicated research results in the main areas of the Artificial Kidney - Chronic Uremia Program. A considerable body of detailed and pertinent scientific and technical information was exchanged between the top staff of 67 currently active research and development projects, the Program's consultants and its staff. It is anticipated that this and future conferences will significantly assist attainment of the Artificial Kidney Program's goals--optimum artificial kidney development, improved clinical methodology, and better patient rehabilitation.

The Proceedings of the Conference will be published and distributed in June 1974.

Project Title:	Conference on Adequacy of Dialysis III and Proceedings
Contract Number:	NIH-74-C-365
Amount:	$30,225
Contractor:	Franklin Hospital
Project Officer:	Dr. Keatha K. Krueger
Other Professional Personnel:	Dr. Robert J. Wineman
Man Years:	Total: 0.30 Professional: 0.15 Others: 0.15

Project Description:

This conference was held in Monterey, California, March 20-22, 1974.

It is well known that present day dialysis therapy is at best partially successful in reversing the several organ system lesions which result from renal failure. Until recently, uremic neuropathy was the only organ system lesion which has been shown to have some quantifiable dependence on the magnitude of dialysis therapy. The relationships to dialysis therapy of many other abnormalities, such as anemia, coagulopathy, osteodystrophy and immunologic deficiencies remain largely undefined. It should be our utmost concern that the dialysis treatment offered be optimal and lead to maximal rehabilitation of the patients. Also, because of the high cost borne by the public, there will be almost certainly a further increase in pressures to reduce cost through use of a less expensive or more efficient dialyzer and reduced treatment time. Rational changes of these treatment parameters will require definition of adequate dialysis therapy.

With the more than ten thousand individuals being maintained by hemodialysis today there is an intense need to provide clinicians with reasonable guidelines for therapy. Unfortunately dialysis time and frequency vary significantly from center to center with relatively little objective data to support one schedule over another.

Where it is possible with presently available data, one objective of the conference was to explore and propose quantitative parameters of treatment to extend to dialysis patients a maximal degree of near-normal physiologic existence and rehabilitation.

Where existing data was inadequate, a second purpose of the conference was to define protocols for the study of end-stage uremic lesions of the dialyzed patient and variables of patient therapy such that quantitative relationships can be established.

The proceedings of the conference will be published in Kidney International.

Project Title:	Conference on Acute Renal Failure
Contract Number:	NIH 73-C-788
Amount:	$19,133.75
Contractor:	Downstate Medical Center
Project Officer:	Dr. Keatha K. Krueger
Man Years:	Total: 0.06 Professional: 0.03 Others: 0.03

Project Description:

A conference was held May 18-19, 1973 at Downstate Medical Center, Brooklyn, New York to update the understanding of the pathogenesis and treatment of acute renal failure and serve as a forum introduction of new information on its management with dialysis, and prevention in transplant patients. Topics covered were pathogenesis, severity and epidemiology, clinical experience, drugs, and acute renal failure following transplantation. Proceedings were published, and appeared in April 1974 (DHEW Publication No. (NIH) 74-608).

Project Title: Workshop on Acupuncture in Rheumatic Diseases

Personnel: Dr. Ronald Lamont-Havers
Dr. Keatha K. Krueger

Man Years:

Total:	0.25
Professional:	0.20
Other:	0.05

Project Description:

Twenty-one participants, representing various aspects of the practice of acupuncture, met to discuss generally the problems associated with the design of control studies and to discuss specifically whether or not rheumatoid arthritis or ankylosing spondylitis were suitable disease entities for a controlled study. Although a specific definitive answer was neither expected nor arrived at, a great deal of useful and practical information emerged. A report of the conference will appear in the open literature this year.

Project Title:	Human Growth Hormone Symposium	
Contract Number:	PH-43-67-1153	
Amount:	$19,272	
Contractor:	National Pituitary Agency	
Professional Personnel:	Total:	0.08
	Professional:	0.04
	Others:	0.04

Project Description:

Approximately 250 people attended this symposium on Human Growth Hormone. The program was very extensive and included sessions on somatomedin, growth hormone hypothalmic control in relation to releasing and inhibitory factors, growth hormone in relation to carbohydrate metabolism and nutrition, structure and synthesis of human growth hormone, the heterogeneity of radioreceptor assays, and clinical studies including therapy.

Proceedings of this symposium will appear later this month (DHEW Publication No. (NIH) 74-612).

Project Title:	Conference on End-Stage Diabetic Nephropathy
Amount:	$7,300
Contractor:	Minneapolis Medical Research Foundation
Professional Personnel:	Dr. Keatha K. Krueger
Man Years:	Total: 0.02 Professional: 0.02 Other: negligible

Project Description:

This conference will be held in Minneapolis, May 23-24, 1974.

Experience in treating end-stage diabetic nephropathy patients is at present limited to relatively few centers in the United States. This conference is designed to examine the problems associated with these particular patients and to disseminate the information that has been gained by the few centers who have been working with diabetic patients in order for involved clinicians to be better able to make rational, informed decisions in managing this problem.

The proceedings of the conference will be published in Kidney International.

Project Title: Second NIAMDD Psoriasis Workshop

Professional Personnel: Dr. Laurence H. Miller
Dr. Keatha K. Krueger

Man Years:

Total:	0.10
Professional:	0.08
Other:	0.02

Project Description:

The Second NIAMDD Psoriasis Workshop was held March 11 and 12, 1974 at NIH. Its purpose was to review current information available regarding the pathogenesis of psoriasis and to draw up a comprehensive research program so that this Institute may effectively plan and coordinate its future psoriasis research activities.

A report of the meeting will be published in the open literature.

Project Title: Workshop on Arthritis Centers

Professional Personnel: Dr. R. W. Lamont-Havers

Project Description:

This workshop was jointly sponsored and funded by the NIAMDD, the Arthritis Foundation, and the American Academy of Orthopaedic Surgeons. The concepts of what components would constitute an "ideal arthritis center" were discussed. A detailed report will be written and will be available to the sponsoring organizations for use in their planning.

Project Title: Cystic Fibrosis Bibliography

Contract Number: NIH 72-2214

Amount: $8,965

Contractor: National Cystic Fibrosis Research Foundation

Project Officer: Dr. Keatha K. Krueger

Man Years:

Total:	0.04
Professional:	0.03
Other:	0.01

Project Description:

The National Cystic Fibrosis Research Foundation is preparing and indexing a comprehensive bibliography dealing with the various aspects of cystic fibrosis in order to make available in one place a complete listing of all available references, to establish an initial comprehensive bibliography which can be kept current by annual updating, and to serve as an educational stimulus to individuals in this and allied health fields.

The citations ultimately will be on magnetic tapes suitable for printing via the Linotron. It is expected to be ready for distribution in the fall.

Project Title: University Group Diabetes Program (UGDP) - Assessment of the Clinical Trials of Oral Antidiabetic Agents

Contract Number: NIH-73-C-213

Amount: $73,723

Contractor: Yale University (for the Biometric Society)

Project Officer: Dr. Keatha K. Krueger (For Dr. G. Donald Whedon)

Man Years:

Total:	0.08
Professional:	0.04
Other:	0.04

Project Description:

A group of biostatisticians has been called together to perform an evaluation of the clinical trials of oral antihyperglycemic agents with reference to their effects on morbidity and mortality in diabetic patients, with particular reference to the vascular complications. The validity and conclusions of the University Group Diabetes Program will be primarily assessed. The report of this group is expected in early summer 1974.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR
PROGRAM ANALYSIS AND SCIENTIFIC COMMUNICATION

ARTIFICIAL KIDNEY-CHRONIC UREMIA PROGRAM

Summary Report

Introduction

The Annual Reports for the previous years provide detailed background on the problem of end-stage kidney disease and elaborate on developments in the modality for its treatment, chronic intermittent hemodialysis. In addition, these reports give a detailed description of the planned and centrally directed contract program of research and development organized at the National Institute of Arthritis and Metabolic Diseases in 1966--the Artificial Kidney-Chronic Uremia Program--which is aimed at development of improved and less expensive dialysis hardware and methodologies and at optimal rehabilitation of chronic dialysis patients. This background material is not repeated here but the reader might wish to familiarize himself with it by perusing the previous reports.

Current Activities

The Artificial Kidney-Chronic Uremia Program endeavors to bring about improvements in artificial kidney apparatus and methodologies, to increase their effectiveness, to decrease their initial and operational cost, and to improve the rehabilitation of patients treated with chronic intermittent dialysis. Optimal development of new, better dialysis apparatus and methods is still hampered by incomplete knowledge concerning the specifics of the uremic syndrome. Hence, knowledge gained from research into the fundamental aspect of the toxic nature of uremia and basic and clinical studies into the long-term effects of uremia and of chronic intermittent dialysis are critical to arriving at a reasonable solution to the overall problem. Thus, the Program not only sponsors hardward improvement *per se*, but stimulates and supports a wide spectrum of research ranging from fundamental studies designed to clarify the toxic nature of uremia and clinical studies on the complications of chronic uremia and maintenance dialysis, to development of improved dialysis or apparatus. In recent years there has been an increasing emphasis on laboratory and clinical studies intended to bring about a greater understanding of the nature of uremia and its major complications.

At present about 70 research and development projects are supported by contracts with universities, nonprofit and industrial research laboratories to give a broad approach to problems of highest priority. Projects are focused on problem areas including clinical trials of more efficient or less costly dialyzers, development of special adsorbents for uremic toxins, studies to elucidate the nature of the aberrant metabolic changes in uremia,

and the maintenance of a national registry of all patients who are being maintained with the aid of dialysis. The Program is also funding studies aimed at isolation and identification of toxic factors which must be removed from the patient's blood, in addition to metabolic wastes and other substances known to accumulate during kidney failure. A large number of research projects is also devoted to the pathophysiology of uremia in an effort to prevent or control complications in patients who have lost their kidney function and are being maintained with the aid of artificial kidneys. Much of the future success in simplifying the treatment of patients with end-stage kidney disease depends on developments in these and other areas of fundamental reserach.

Accomplishments

Decreasing Treatment Time. One important aim of the Artificial Kidney Program is to change dialysis methodology or dialyzer design which would reduce the overall weekly dialysis time (which now amounts to 20 or more hours divided into three treatment episodes per week). This would decrease the high cost of this treatment and enable patients to devote more of their time to productive work or recreation. During the last year several investigators have successfully introduced innovations utilizing more than one standard dialyzer or testing new dialyzers which have a considerably enlarged membrane area and thus purify more blood per unit of time than conventional dialyzers. Thus far these experimental treatments have involved a relatively small number of patients but they have demonstrated that in the future it will be possible to shorten dialysis treatment time for most patients.

Improved Dialysis Membrane. At the present time several novel membranes which have a greater permeability to substances that must be removed from the patient's blood are being tested. The aim is to decrease the time spent on dialysis and, if possible, to induce more thorough removal from the blood of uremic toxins which have a molecular weight considerably higher than urea or creatinine. There are indications that substances which are not yet characterized, who molecular weight ranges from 1300 to about 5000, may be responsible for some of the serious toxic manifestations in uremia. "Tight" membranes do not permit a rapid flux of such substances in the dialyzer. It is hoped that more permeable membranes will lead to effective dialysis devoid of complications, namely anemia, peripheral neuropathy, and osteodystrophy (decreased bone density).

A New Prosthetic Arteriovenous Shunt. Institute grantees and their associates at the University of Minnesota Hospitals have devised an implantable, compact prosthetic arteriovenous blood access shunt that offers several advantages over standard shunts and passages, notably faster blood flow and less clotting, and very few complications. The new

device, because of its shorter length and decreases resistance allows three times faster blood flow than the standard Quinton-Scribner shunt. The faster flow prevents blood sludging and clotting, decreased incidence of infection and trauma, and provides for prolonged shunt survival. The new shunt has been implanted in many patients previously unable to maintain functional standard shunts, and results have been so encouraging it has been adopted as standard at the University of Minnesota Hospitals.

Success in Preventing or Controlling Uremic Osteodystrophy. The results of studies of the last several years have recently converged to the point where it is now possible in well supervised patients to prevent or control uremic osteodystrophy, a common and serious complication of chronic renal failure. A large percentage of patients maintained with repeated dialysis suffer from this characteristic bone demineralization which weakens the skeleton and makes it prone to fractures. In many cases, the excess calcium and phosphate liberated from the skeleton may be deposited in soft tissues in the joints, the eye, and even heart muscle.

The bone weakening is the end result of a combination of factors. The progressively decreasing kidney function permits retention in the body of excessive phosphate; at the same time, impaired vitamin D activity prevents adequate intestinal absorption of dietary calcium. As a result, in an effort to increase calcium blood levels to normal, the parathyroid gland secretes excessive amounts of parathyroid hormone which causes a mobiliation of calcium (and phosphate) from the bone in an effort to compensate for the low blood calcium levels.

Research workers working individually and in concert have not proposed novel, clinically effective methods of prevention.

An Institute grantee has demonstrated that vitamin D is not biologically active in the body but a secondary derivative of this vitamin (1,25-DHCC) is. A primary derivative (25-HCC) is formed from vitamin D in the liver and must still be further modified in the normal kidney to the secondary derivative, 1,25-DHCC. This is the active substance which stimulates intestinal absorption of calcium from the diet, and calcium mobilization from and deposition in bone. In kidney failure, because of the lack of kidney tissue for this conversion of vitamin D, a profound impairment of calcium matabolism takes place. The Institute has taken steps to procure through chemical synthesis quantities of 1,25-DHCC for distribution to research scientists and for eventual therapeutic trials.

Other research contractors have demonstrated that the overactive parathyroid and calcium abnormalities in chronic renal failure can be avoided in dialyzed patients by restriction of phosphate intake. This is done by adding phosphate-absorbing substances to the diet. It has also been found that elevation of the calcium concentration in the dialysis bath will obtain higher calcium blood levels and counteract the tendency for secondary hyperparathyroidism. A combination of both phosphate restriction and blood calcium increase, if started early enough, has been shown effective in preventing the body dystrophy of chronic renal failure.

Use of a Gastrointestinal Urea Binder. Studies have now proved conclusively that a significant portion of the urea burden of patients in kidney failure can be removed from the body through the oral administration of polyaldehyde substance which is an oxidation product of edible starch ("oxystarch"). The idea for a specific sequestering agent to bind urea (or its intestinal breakdown product, ammonia) in the gastrointestinal tract and carry it out in the feces, came from an investigator in Italy whose research was subsequently sponsored by this Institute. Now large-scale, well-controlled clinical trials in the United States have demonstrated that "oxystarch" will remove significant quantities of urea and ammonia nitrogen resulting in a marked drop of the blood urea nitrogen concentration. This substance also sequesters and eliminates potassium; this is likewise desirable since potassium accumulates in the uremic patient to dangerous levels which may cause cardiac irregularities or even cardiac standstill. A followup study is planned to determine to what extent administration of oxystarch permits a decrease in the frequency or length of dialysis.

New Developments in Peritoneal Dialysis. Research contractors are continuing developments in peritoneal dialysis equipment. They have designed and tested a new, fully automated, closed cycle peritoneal dialysis machine which has significantly reduced the cost of treatment because it prepares its own sterile and pyrogen-free dialysate solution. The solution is periodically introduced into the abdominal cavity and emptied therefrom, carrying with it the toxic waste products of metabolism. This equipment has eliminated to a large extent the chances for inadvertent infection. The system prepares sterile pyrogen-free water automatically from tap water through a new process, reverse osmosis filtration. Trials in home dialysis have shown the mothod is effictive and inexpensive.

With these developments, peritoneal dialysis - at one time almost abandoned in favor of hemodialysis because of a number of drawbacks - has now become an acceptable alternative. In fact for some patients it may become the preferred treatment. These include patients who have lost, over the years, all accessible blood vessel sites for hemodialysis connections and candidates for renal transplantation who expect surgery within a brief time.

Another group of investigators has succeeded in a novel technique for peritoneal dialysis which had been used previously only in experimental animals. This new peritoneal dialysis technique involves the regeneration of dialysate by circulating it through a mixed group of sorbents which specifically bind the toxins washed out of the peritoneal cavity. This system avoids the necessity for large volumes of sterile, pyrogen-free dialysate containing fairly large quantities of different chemicals, the most expensive ingredients in standard peritoneal dialysis.

Such a system, once perfected and thoroughly tested, also will be relatively simple and inexpensive for peritoneal dialysis in the emergency treatment of acute kidney failure in hospitals.

National Dialysis Registry. The National Dialysis Registry, organized and maintained by the Institute, shows that in the United States about 12,000 patients are kept alive with the aid of artificial kidneys. The annual mortality of those on chronic dialysis is approximately 10% during the first year and from 7 to 9% during subsequent years. The Registry data indicate that the most frequent cause of death appears to be accelerated degeneration of the blood vessels akin to the accelerated atherosclerosis found in the large blood vessels of diabetic patients. Over 60% of long-term dialysis patients die of heart disease and strokes. Consequently the Artificial Kidney Program will undertake studies of the abnormal fat and carbohydrate metabolism in uremic patients which may be relevant to this mortality factor.

Working Conference on Adequacy of Chronic Dialysis

The extension of Medicare coverage to provide dialysis when needed for about 90% of all workers and their dependents in the United States (the population covered by Social Security Act amendments) will require dialysis facilities and trained technicians to treat an estimated dialysis population of 40,000 within a few years. Even more important, in the long run, will be the need to improve the physical and mental well-being of these patients to the point where they can lead normal and productive lives. To this end the dialysis treatment must not merely be "life-saving" but also safeguard the quality of the patients' remaining years.

Assessing the quality of care of dialysis patients is an extremely complex problem. Lack of universally acceptable criteria which can be objectively measured results in the reliance upon physicians' impressions of each patient's sense of well-being, his ability to lead a productive and enjoyable life, and his freedom from uremic symptoms. The problem is complicated by the fact that the many different dialysis centers utilize different equipment and treatment regimens, with no agreement on optimal treatment. To aid in the search for uniform criteria, the Institute, in the Spring of 1972, sponsored a preliminary conference at Bethesda. A large conference was held early in 1974 to explore and propose qualitative and quantitative parameters of treatment to extend to dialysis patients a maximal degree of near-normal physiologic existence and rehabilitation. Approximately 200 leaders and experts in the field of dialysis participated in this landmark symposium to propose benchmarks of adequacy for the new national dialysis treatment program.

Conferences, Publications, and Scientific Communication

The Institute regularly organizes symposia and workshops on critical problems and opportunities in kidney disease research. The most recent of these conferences was devoted to Acute Renal Failure. Others scheduled for 1974 include a symposium on the Optimal Treatment of Diabetic Patients with End-Stage Kidney Disease and a conference on the Endocrine Functions of the Kidney. Each year, also, the Institute brings together the research contractors of the Artificial Kidney Program with staff and

consultants for a comprehensive meeting at which all ongoing work is reported and discussed. Extensive illustrated proceedings, describing the most recent advances, are published and broadly distributed.

The Institute also distributes to concerned groups and individuals thousands of pamphlets descriptive of kidney disease, dialysis, and kidney transplantation, and responds to inquiries on specific related topics.

Outlook for Kidney Disease and Research and Development

Research conducted and supported by the National Institute of Arthritis, Metabolism, and Digestive Diseases has added much to the understanding and treatment of kidney disease. Clearly this review of investigation and progress in the broad area of kidney disease, artificial kidneys, and renal transplantation is not all-encompassing. Nonetheless, the variety and fruitfulness of the Institute's efforts and the stimuli to further investigation are apparent. Investigations such as those discussed in this report have increased our ability to cope with the problem of kidney failure. Continued research efforts are greatly needed. The effort to reduce kidney disease must include all of the available components - research, prevention, treatment, and education. The total program is aimed at all the primary kidney diseases as well as at end-stage kidney failure. Combined with fundamental research, which is the essential underpinning, this program should result in maximal benefits in the saving of lives and prevention of disease.

CONTRACTOR: Albany Medical College of Union University

AMOUNT: $31,046

TITLE: Electronic EEG Frequency Analysis for Evaluation of Uremia

OBJECTIVES: The contractor's objective is to attempt to devise an electronic system which would enable detailed and sophisticated analyses of the EEG in uremia and to attempt to use it as a tool to predict the severity of the clinical status of the patient.

MAJOR FINDINGS:

The program has developed the proposed electronic system and has shown the EEG's of patients dialyzed three times per week are generally better than those of patients dialyzed twice a week.

Contractor: Amicon Corporation

Amount: $88,792

Title: An Improved Hollow Fiber Hemodialyzer

Objectives: The contractor's objectives are: 1) full clinical maintenance on two patients employing a six-month cross-over protocol. Continuous monitoring of readily-accessible and generally-accepted physiological parameters is planned. Some additional emphasis will be given to quantification of therapy via newer techniques; 2) short-term exposure to a larger patient population, including corrobatory testing outside the University of Pennsylvania; 3) investigation of single patient and cross-patient reuse. The most promising of the 2.00 M^2 designs thus far evaluated will be employed. This is a 10,000-fiber dialyzer with 13" of active fiber length, 2.23 m^2 active area, 42.1 ml priming volume (exclusive of end caps), and fiber density of 4,820/in^2. Preliminary *in vitro* performance of this model shows a sodium chloride clearance of 160 ml/min. at Q_B= 200 ml/min. and Q_D= 500 ml/min.

Major Findings: The goal of this program is to develop an improved hemodialyzer based upon Amicon's polysulfone, PMD hollow fibers. The polysulfone fibers are produced via a simple wet-spinning process, and require no chemical treatment after initial preparation. They are strong (burst strength exceeds 100 psi) and easy to process. Membrane mass transfer resistance is equivalent to Cuprophane^R PT-150 for lower molecular weight species and significantly lower than Cuprophane^R for intermediate and higher molecular weight molecules. Blood ultrafiltration rate is about three times that of cellulosic hollow fibers. Since they are not hydrogels, the PMD fibers may be dried before and after Potting and may be sterilized by ethylene oxide, which would be appropriate for initial manufacture and dry shipment, or autoclaved, which might be beneficial in reuse.

During the past year, dialyzers varying in area from 0.4 M^2 (4,000 fibers) up to 2.0 M^2 (20,000) fibers have been designed, fabricated and characterized. Using the smaller 4,000-fiber dialyzers, the minimum fiber packing density for satisfactory reproducible mass transfer was determined. Practical, high-yield techniques for assembling tight-packed units were developed. Single solute *in vitro* characterization was completed and found fully satisfacotry. *In vivo* studies have started.

Proposed Course: This contract will be completed this year.

CONTRACTOR: Avco-Everett Research Laboratory, Ind.

AMOUNT: $99,410

TITLE: Continued Study for Blood Flow Considerations to Minimize Thrombosis in Artificial Kidney Systems.

OBJECTIVE: Thrombus formation on a foreign surface is recognized to be a complex phenomenon dependent upon surface chemistry, hemodialysis and blood chemistry. The contribution of each parameter and their complex interrelationship must be understood if an understanding of the mechanism of thrombosis and its prevention in prosthetic devices is to be attained.

MAJOR FINDINGS: Work completed to date under this contract includes an investigation of thrombus formation in separated flows, and has led to the study by direct observation of the formation and growth of unattached platelet aggregates and their embolization from a separated flow region. Some studies have also been made of the formation of attached or surface aggregation within the separated flow regions. Such aggregation occurs under differing flow conditions from that in which floating aggregates form. During the present contract year, a study of thrombus formation on controlled polyurethane test surfaces having systematically varied molecular surface properties has been initiated. The studies are being performed using test surfaces having well characterized surface properties under controlled flow conditions, and measured hematological parameters.

CONTRACTOR: Avco Everett Research Laboratory, Inc.

AMOUNT: $46,808

TITLE: Development of Avcothane Cannulae

OBJECTIVE: The objective of this contract is the development and testing of cannulae made of "Avcothane."

MAJOR FINDINGS: The shortcomings of 1st-3rd Generation (Gen.) Avcothane shunts which were observed in earlier caprine implantations have been alleviated in 4th-6th Gen. shunts. While retaining Avcothane blood contact surfaces, a porous silicone rubber (MAA) wash coating found to promote tissue adhesion in vivo, covers the outer surface of the tubing section of 4th Gen. shunts. To encourage intimal adhesion, in 6th Gen. shunts, the same coating also covers the tip outer surface except the very end which is Avcothane. According to the experimental protocols established earlier (i.e. no cuffs or buttons attached to cannulae to prevent ascending infection, no prophylactic antibiotics or systemic heparinization postoperatively, etc.) so far a total of 22 exteriorized implantations of 4th-6th Gen. Avcothane shunts were performed in the carotid artery/jugular vein position of goats. These cannulations were interspersed with 4 identical implantations using Silastic/ Silastic-tipped control shunts. As compared to the mean patency of 1st-3rd Gen. shunts of 12 ± 5 days, 4th-6th Gen. shunts are presently at 23.3 ± 13 days. The major failure mode of 4th-6th Gen. shunts lasting for more than 7 days was irreversible occlusion, with no cases of infection which is indicative of the barrier effect of the MAA outer coating. Endothelial tissues, particularly with 6th Gen. shunts showed small, if any, intimal damage. The majority of implanted 4th-6th Gen.shunts occluded irreversibly on the venous side which resulted from hemmorhage from capillary feeder vessels. This mode of failure is entirely different from that of Silastic control shunts, all of which but one occluded on the arterial side by an organized thrombus developing into a flap-type "choke valve". The temporary increases in WBC levels and parallel decreases in platelet and arterial flow levels observed with Silastic controls could not be detected with implanted 4th-6th Gen. Avcothane shunts.

PROPOSED COURSE: In view of the increase attained in the mean patency of 4th-6th Generation (Gen.) Avcothane cannulae which resulted from a systems-type approach to factors affecting in vivo shunt performance, further optimization of these cannulae will be performed by using the same type of approach and with particular attention to the difference in the requirements of arterial and venous cannula/vessel junctions. Shunt evaluations will be performed with exteriorized cannulae in the caprine model, and shunts will consist of combinations of 4th and 6th Gen. cannulae.

In a parallel effort and in preparation to conceivable human trials, 4th-6th Gen. shunts having tips suitable for the smaller human vessels will be prepared and implanted into the femoral vessels of goats which are of the appropriate size. As inherent controls, the same animal will be also femorally cannulated on the other side with Silastic/Teflon-tipped shunts. Both Avcothane and Silastic control shunts will be buried to establish the superiority of either type in the absence of uncontrollable factors.

In view of the results attained with the porous silicone rubber wash coating of cannula outer surfaces, and the fact that this coating can be independently applied to other cannulae which are used presently in patients, the tissue interfacing properties of this coating will be optimized and tested in goats such that the coating should be ready for human trials independently within the next 6-8 months.

CONTRACTOR: Battelle Memorial Institute

AMOUNT: $97,810

TITLE: Feasibility of Microencapsulated Detoxicants for Removal of Nitrogenous Metabolites via Oral Ingestion.

OBJECTIVES: Development of microcapsules capable of irreversibly binding urea, ammonia and other toxic metabolites.

MAJOR FINDINGS:

The Battelle group have found that chemical binders of urea are inadequate and the most rewarding area of investigation is to develop methods to stabilize urease and bind this enzyme to polymers which can then be encapsulated. The urea would then react with the urease and aspartase and be converted to ammonia which then could be adsorbed by resins containing sulfonic acid groups which are also encapsulated. The stabilized urease has been encapsulated as well as the resins containing sulfonic acid. Their microencapsulated system has shown to be effective in vitro and in azotemic animals. The concentrations of selective nitrogenous metabolites in various regions of the dog small intestine were determined in control and azotemic animals and correlated to blood levels of creatinine urea, and uric acid.

PROPOSED COURSE:

This project has been terminated.

CONTRACTOR: University of California

AMOUNT: $40,871

TITLE: Nutritional Supplementation in Children with Chronic Renal Failure

OBJECTIVE: The project's objective is the examination of the growth of children with kidney disease who are growth retarded.

MAJOR FINDINGS: Two infants with severe renal insufficiency have been followed eight months and three months using Ross Renal Formula and growth in height, weight and cell mass has occurred. Two other children have been receiving calorie supplements since age 2; and four children with symptomatic uremia and acute wasting have been given intravenous essential amino acids and glucose at night as well as developing with the nutritionist some "homemade" formulas. These measures have increased total calorie intake and been associated with a lower SUN, increased weight and possible increased weight, cell mass and body fat.

CONTRACTOR: University of California, Los Angeles

AMOUNT: $93,000

TITLE: Nitrogen Metabolism in Uremia

OBJECTIVES: The objectives of this investigation are to study the metabolism of amino acids and their products and the enzymes which mediate the metabolism of these substances in uremia. It is believed that this information may help to define causes of uremic wasting and uremic toxicity, and may provide means of assessing optimal dietary treatment and dialysis regimens.

MAJOR FINDINGS: Histidine appears to be an essential amino acid not only in uremic patients but in normal man as well. Phenylalanine metabolism is abnormal in uremia. Activity of both monoamine oxidase and diamine oxidase is elevated in plasma of chronically uremic subjects.

PROPOSED COURSE:

1. Continue to analyze amino acid content of plasma, muscle and liver from uremic patients.

2. Determine the dietary histidine requirements in normal and uremic subjects.

3. Determine the abnormal by-products of phenylalanine metabolism in uremia and perform toxicity studies.

4. Determine the locus of abnormal amine oxidase activity.

CONTRACTOR: Cedars-Sinai Medical Center

AMOUNT: $192,805

TITLE: Osteodystrophy and Divalent Ions in Kidney Failure

OBJECTIVE: Perform a systematic and comprehensive study to evaluate the mechanisms leading to deranged divalent ion metabolism in renal failure.

MAJOR FINDINGS: Resistance to the action of PTH has been demonstrated with the use of PTH infusion and EDTA infusion. The mean elevation in serum calcium seen with PTH infusion was significantly reduced in patients with early, moderate and severe renal failure. Only half of the dialysis patients had normal responses. Subtotal parathyroidectomy did not improve the response. Following EDTA infusion the serum calcium of uremic patients failed to return to normal after 30 hours.

Administration of $1,25(OH)_2D_3$ (0.63-2.7 mg/day) caused a marked increase in calcium absorption from the gastrointestinal tract. The greatest improvement was seen in absorption from the proximal small bowel.

PROPOSED COURSE: The emphasis on the continued work will be in studying the causes for the decreased absorption of calcium from the gastrointestinal tract. Modes of therapy will include large doses of oral calcium, $1,25(OH)_2D_3$, 5,6-trans-$25OHD_3$, 1-α-OHD_3, dihydrotachysterol. Calcium balance and bone biopsies will be followed.

Contractor: Cedar-Sinai Medical Center

Amount: $90,000

Title: Sorbent-based Regenerative Peritoneal Dialysis System

Objectives:

To develop a peritoneal dialysis system in which the dialysate is circulated through a sorbent cartridge for regeneration.

Major Findings:

The feasibility of the concept has been demonstrated using the same sorbent cartridge presently in use in the REDY^{R} system.

Proposed Course:

The system will be optimized and a prototype will be built. The contractor will conduct detailed sterility studies and preliminary clinical studies will be initiated with uremic patients undergoing peritoneal dialysis.

CONTRACTOR: Cedars-Sinai Medical Center

AMOUNT. $36,253

TITLE: Investigations into the Pathophysiology of Dialysis Disequilibrium Syndrome

OBJECTIVES: To determine the alterations in brain intracellular pH, brain water and electrolyte metabolism and alterations in skeletal muscle in acute and chronic renal failure and the effects of hemodialysis on these parameters. These investigations are undertaken in an effort to elucidate the pathophysiology of the dialysis disequilibrium syndrome and its possible prevention.

MAJOR FINDINGS: During rapid hemodialysis the osmolality of the dog's brain increases, but the increase is not due to measured solute (Na^+, K^+, Cl^-, Ca^{++}, Mg^{++}, urea) The investigators postulate the generation of idiogenic osmoles as a consequence of an observable drop in brain intracellular pH. Preliminary studies suggest that the drop in intracellular pH and increase in total brain solute content can be prevented by the addition of glycerol to the dialysate bath.

PROPOSED COURSE: They plan to continue their efforts to prevent dialysis disequilibrium by the addition of glycerol to the dialysate. If this mode of therapy proves to be non-toxic, this may lead to clinical testing.

Contractor: Celanese Research Company

Amount: $99,133

Title: Preparation and Evaluation of Optimized Hemodialysis Membrane

Objectives: The contractor's objectives are to: 1) fabricate 4-6 cellulose acetate (CA) membrane variants in quantities suitable for meaningful clinical trial; 2) investigate promising new materials with a view toward eventual production level efforts where indicated; 3) examine experimental membranes for their promise as possible dialysis membranes (electret membranes, PBI membranes and composite structures).

Major Findings: Improved cellulose acetate membranes for hemodialysis have been prepared in these laboratories. The important variables associated with the fabrication of these membranes have been defined and a number of variants have been produced from commercial polymer grades in several thousand foot quantities. *In vitro* characterization of the first of these membranes is now nearly complete. Dialysis permeabilities were measured in an NBS cell and the permeability of this membrane to "middle molecules" was found to be outstanding (4.5 - 13X relative to Cuprophan). The membrane represents a clearcut improvement over previous CA membranes in terms of transport, blood compatibility, and linearity to pressure response. Indeed, its blood compatibility has been shown to be distinctly superior to PT-150. The feasibility of glycerinization (to obviate the need for a wet membrane) has been demonstrated and several leads to membranes with hydraulic permeabilities of 1-3X Cuprophan have been established. Finally, a preliminary economic projection suggests CA membranes would be cost competitive with Cuprophan, with raw materials amounting to less than $.05/m^2$.

Proposed Course: This contract will be continued to supply experimental membranes for clinical evaluation.

Contractor: Cleveland Clinic Foundation

Amount: $50,000

Title: Development of New Concepts in Blood Access Devices

Objectives: Development of fistula shunts using thromboresistent biolized materials.

Major Findings:

The contractor has demonstrated that his puncturable extra-cutaneous shunts made of biolized materials have a patency three to four times that of standard commercially available silicone rubber shunts.

Proposed Course:

Studies with shunts implanted in sheeps will be continued. The design and fabrication of shunts for human patients will be initiated.

Contractor: Cleveland Clinic Foundation

Amount: $57,000

Title: Development of the Envelope Kidney

Objectives: Using the design of the Coiled Envelope Kidney developed in previous contract work sufficient units will be constructed to carry out in vivo animal studies (at least five dog dialyses), and initial clinical trials (approximately 200 patient dialyses). Adequate quality control procedures for the Envelope Kidney production will be set forth and liaison with commercial organizations in order to develop a mass production model including additional cost studies, and establishing the necessary quality control procedures in assembly, packaging, and sterilization will be carried out. Additional clinical dialyses (approximately 540) using the finalized model and in vitro studies to determine the function of the membrane supporting scheme on higher molecular weight solute mass transport characteristics will be carried out.

Major Findings: The initial concept being developed is that a sterile package of a supported tubular membrane with blood ports attached can be provided to a patient for self-winding of a coil dialyzer at a cost reduction compared to commercially available coil dialyzers. The primary tasks of the past work have been to show the feasibility of the concept and to design and construct the finalized components. Preliminary in vitro studies showed the feasibility of the concept and further parametric studies were carried out to characterize the coil for reproducible construction by the patient. These in vitro studies indicated that performance with the Coiled Envelope Kidney is at least comparable to presently available dialyzers. The effect of winding tension on coil construction was quantitated. In vivo dog dialyses have indicated no particular problems relating to the design of the Coiled Envelope Kidney. A finalized design of the blood manifold and related equipment has been chosen and components are being constructed. The primary membrane support structure has been chosen based on strength characteristics of available plastic nettings. A winding machine for patient use has been designed and constructed which incorporates a tension controlling device which can be pre-set at the desired tension for coil winding. The time requirement for assembly of the Coiled Envelope Kidney is about two minutes by one person. A preliminary packaging scheme of the Coiled Envelope has been designed and is being used. Sterilization checks of the Coiled Envelope in a commercial gas sterilizer have not indicated any particular problems relating to sterilization of this package. A present cost accounting indicates the Coiled Envelope can be supplied to the patient at $7-8.

Proposed Course: This contract will be completed this year.

Contractor: Esso Research and Engineering Company

Amount: $22,000

Title: Liquid Membrane Capsule System for the Treatment of Chronic Uremia

Objectives:

To develop a liquid membrane to encapsulate reactants to be used as toxin traps in the treatment of chronic uremia.

Major Findings:

The liquid membrane formulation has been demonstrated to be effective in removing ammonia from both solutions and synthetic gut fluid. Effective formulations have been used to encapsulate 10 normal hydrochloric acid or 50 weight percent sulfuric acid.

The mobility and stability of the liquid membrane capsules have been successfully tested in dogs.

Proposed Course:

The contractor will attempt to increase the rate of toxin removal using an oil phase additive with higher solubility for toxins, or an oil formulation with lower viscosity. He also will attempt to increase the capacity for toxin removal and the stability of the liquid membrane.

CONTRACTOR: Georgetown University

AMOUNT: $15,000

TITLE: New Approaches to the Design of Chronic Dialysis Cannulae for the Prevention of Infection and Clotting

OBJECTIVE: The objective of this contract is the investigation of parameters influencing cannula patency and the design of less thrombogenic and longer lived blood access devices.

MAJOR FINDINGS: During the period of this contract fourteen cannula designs have been studied. Each of these has been made with several modifications and these have been evaluated according to duration of patency, the incidence of thrombus formation, fibrosis at the cannula tip, cannula dislodgment and infection.

Studies of the cutaneous cannula junction have been made to determine the optimal shape and size of the cannula exit configuration. These studies have demonstrated that these cannula can give prolonged patency in dogs even when the internal diameter of the shunt is between two and three millimeters in diameter. Velour covered soft tips have given patency rates in dogs between 60 and 90 days in the majority of cases. Duration of other types of cannulae in dogs tend to be in the range of 20 to 30 days. During the last year clinical studies have been carried out with further modification of the basic cannula design which uses the cutaneous cannula junction previously developed.

Contractor: Gulf South Reserach Institute

Amount: $27,999

Title: Membranes and Materials Evaluation: Permeabilities, Physical and Mechanical Properties

Major Findings:

Detailed evaluation of four flat sheet membranes has been completed. A lot of cuprophan was evaluated and will be retained as a reference membrane.

Proposed Course:

The evaluation of potential hemodialysis membranes, capillary fibers and materials in relation to their permeability, physical and mechanical properties will be continued.

CONTRACTOR: Good Samaritan Hospital, Portland, Oregon

AMOUNT: $24,242

TITLE: Platelet Function as an Indicator of Adequate Dialysis

OBJECTIVES: This study is undertaken in an attempt to correlate platelet function to dialysate flow-rate and the number of hours of dialysis.

MAJOR FINDINGS: Investigations are in a preliminary phase and no conclusions can be drawn at this time.

PROPOSED COURSE: Continue investigating the relationship between clinical status, blood chemistry and platelet function with constant dialysate flow-rate, reduced dialysate flow-rate and reduced dialysis time.

CONTRACTOR: Jefferson Medical College

AMOUNT: $105,314 (two years)

TITLE: Amine Metabolism in Uremia

OBJECTIVES: Study the metabolism and toxicology of aliphatic amines in uremia with emphasis on the role of intestinal bacteria in the generation of these amines.

MAJOR FINDINGS: The bacterial counts found within the duodenum and jejunum of severely uremic patients are significantly higher than those of normals or mildly uremic patients. These high counts are due largely to the presence of streptococci and bacilli (neither identified as yet.)

Serum Dimethylamine and trimethylamine concentrations are higher in uremics than normals. Intestinal DMA concentrations are higher than serum concentrations. The reverse is true for TMA.

PROPOSED COURSE: DMA and TMA concentrations will be correlated with the severity of uremia. The abnormal bacterial flora will be identified.

CONTRACTOR: Mayo Foundation

AMOUNT: $38,659 - April 11, 1973 to April 10, 1974

TITLE: Metabolism of Creatinine and Guanidine Compounds

OBJECTIVE: The aim of this study is tripartite: 1) to define more clearly the relationship of the readily determined plasma creatinine concentration to true renal function and to symptoms of reduced renal function; 2) to identify the metabolites, metabolic pathway, and site of metabolism of the endogenous (biochemically active and potentially toxic) products of creatinine metabolism; 3) to determine the physiological significance of these metabolites of creatinine, when identified, and of guanidinosuccinic acid (GSA), by monitoring their concentration during the progression of the symptoms and of remissions of symptoms in patients subjected to treatment regimens.

MAJOR FINDINGS: Balance studies have been performed with eight patients with reduced renal function after administration of isotopically labelled creatinine ($^{14}CH_3$ creatinine and/or ^{14}carbonyl-creatinine). Thirteen to sixty-four per cent of endogenously formed creatinine was cleared by another route than the commonly assumed renal route and then metabolized. This quantity of 190-715 mg creatinine per day is represented by an unidentified metabolite which appears in the plasma, cannot be recovered from the ion exchange column, but is present in the hemodialysate of the patient. Those metabolites identified include: CO_2, methylamine, N-methylamine, N-Methylhydantoin, sarcosine, and a minor "unknown".

PROPOSED COURSE: Creatinine a metabolite and the site of metabolism of creatinine will be studied. Standard isolation characterization techniques including hydrolysis, reduction, and oxidation followed by chromatography have been and will continue to be used. Adequate amounts of material, especially the unknown have been obtained by dialysis, to facilitate appropriate identification. The metabolite formation *in vitro* will be studied by aerobic and anaerobic incubation of $^{14}CH_3$ creatinine and its products with intestinal contents obtained by intubation (with a multiluminal catheter) of patients with diminished renal function. If it appears that the enzymic activity resides in the intestinal mucosa, tissue specimens taken at *post mortem* examination will be utilized for further *in vitro* studies. It is hoped that this data can be utilized in describing the site of metabolism in the gut as well as the metabolic pathway of creatinine degradation by gut flora. Appropriate control studies will be performed with material from non-uremic patients. Emphasis will be placed solely on biochemical studies of creatinine and its metabolites in uremia.

CONTRACTOR: Mayo Foundation

AMOUNT: $130,444

TITLE: Value of Maintaining Parathyroid Hormone Suppressive Calcemia in Prevention of Bone Disease and Abnormalities in Calcium Homeostasis in Patients on Long-Term Hemodialysis.

OBJECTIVES: To examine the alterations in calcium metabolism and parathyroid function as they relate to the development of renal osteodystrophy, in particular dialytic bone disease.

To examine in detail 25 OH-D_3 metabolism in patients with chronic renal insufficiency.

MAJOR FINDINGS: Dialysate Ca level and plasma phosphorus have been identified as major factors contributing to the secondary hyperparathyroidism associated with chronic renal insufficiency. Serum immunoreactive PTH (measured by antibody GP1M) can be lowered toward normal by controlling plasma Pi ($<$ 6 mg/dl) with the use of oral phosphate binders and dialyzing patients against a dialysate Ca concentration of 7 - 8 mg/dl. The high dialysate Ca is necessary for positive Ca balance, i.e. to counter balance the decreased oral intake and increased fecal losses.

Kinetic studies of 25OHD_3 metabolism have begun but the data are too small for interpretation.

PROPOSED COURSE: The investigator plans to continue the 25OH-D_3 kinetic studies. Methods for separating D_2 from D_3 have been devised. In addition an assay for 1,25 $(OH)_2D_3$ is being developed.

CONTRACTOR: Mayo Foundation

AMOUNT: $88,138

TITLE: Uremic Neuropathy

OBJECTIVES: The presence and severity of peripheral neuropathy in patients on various hemodialysis treatment schedules will be evaluated to find the most efficient and economical management to prevent or minimize this complication.

A touch-pressure instrument has been developed and appears to be a sensitive indicator of uremic neuropathy. No deterioration of peripheral nerve function has been found in patients dialyzed once a week while maintained on specific fluid and dietary restrictions.

PROPOSED COURSE:

a) Continue the assessment of infrequent dialysis on nerve functions.

b) Continue work on histologic measures of nerve damage.

c) Assess plasma lipoproteins and myelin composition in uremia.

d) Develop an animal model of uremic neuropathy.

Project Title: Computer Data Bank to Assess Adequacy of Dialysis

Contract Number: 72-2225

Amount: $80,000

Contractor: Minneapolis Medical Research Foundation

Project Officer: Dr. Keatha K. Krueger

Project Description:

The first stage of the low flow-high flow dialysate study has been completed and will be continued for a longer time with a few patients in order to complete the study with sufficient data for complete statistical evaluation, with the goal of being able to recommend without reservation that Q_D 200 can be used without adverse effects on a dialysis patient.

A study of the brain electrical activity of dialysis patients exposed to various modes of dialysis will be initiated. This study will include an in-depth evaluation of brain wave activity, predialysis and post-dialysis, in patients who are being exposed to large surface area dialyzers with both Q_D 500 and Q_D 200 dialysate flow rates. The EEG's will be recorded, transferred to magnetic tape and analyzed by spectral analysis.

The low flow-high flow dialysate study was performed as previously described with 22 patients completing the study. Dialysate flow rate of 200 cc/min in D4 Kiil dialyzers was not shown, in the three-month span of the study, to adversely affect the dialysis patient as determined by a large number of measures on relevant parameters. The results of the study on the effects of Decadurabolin administered three times per week in a group of patients on regular hemodialysis suggests that the drug has a twofold action, stimulation of erythropoiesis and an anabolic effect. The computerized data system was also further refined.

Contractor: University of Minnesota

Amount: $43,522

Title: Fluid Dynamics of Blood Cells

Objectives:

1. Analysis of blood velocity profiles in narrow channels using a 3-D microscope system.

2. Study of hematocrit skimming layers using fluid conductivity measurements.

3. Couette skimming layer force evaluation.

4. Analysis of flow enhanced diffusion coefficients using the Taylor Dispersion Method.

5. Studies of formed element behavior following wall contact using electron and phase microscopy.

Major Findings: Platelet deposition studies involve the direct study of platelets from platelet rich plasma depositing in the porous surface of the Couette flow apparatus and from fresh whole blood in the Petschek cell equipped with ultrafiltering membrane. To facilitate the study of platelet deposition in kidney machines, a clinical ultrafiltration meter has been designed and is being developed.

Proposed Course: Blood studies and the development of the ultrafiltration meter will be continued.

CONTRACTOR: University of Missouri

AMOUNT: $29,504

TITLE: Adherence of Blood Components to Artificial Kidney Membranes

OBJECTIVES: The purpose of this project is to study the effects of hemoperfusion on dialysis membrane performance.

MAJOR FINDINGS: Despite significant protein and cellular deposits on membrane surface, coils maintain their transport properties as long as they are not air rinsed so that multiple use is no problem. However, hollow fiber kidneys show significant deterioration in function after single use, so that reuse of hollow fiber kidneys requires careful monitoring of artificial kidney function.

PROPOSED COURSE: The direction of investigation has been changed slightly to examine the question of anti-nuclear antibody formation during artificial kidney re-use and mulitple exposure to cellular debris.

Contractor: University of Naples

Amount: $17,500

Title: Studies on Amino Acids in Uremia

Objectives:

To study selected facets of amino acid metabolism in chronic uremia with emphasis on phenylalanine, tryosine and histidine. To establish the true essential amino acid requirements in chronic uremia [which differ from those of normal man]. To investigate the feasibility of special diets for uremic patients based on keto acid analogues of the amino acids essential for maintenance in uremia. To establish the anemia-ameliorating action of histidine in uremia.

Major Findings:

The principal investigator has demonstrated that in chronic uremia the ordinarily nonessential amino acid histidine is indeed essential. Histidine supplementation improves uremic anemia and nitrogen balance. Preliminary studies have indicated that uremic patients can utilize the keto acid analogues of essential amino acids in endogenous protein synthesis. It is of particular interest to note the specific action of histidine in improving hematocrit of uremic patients. This is consistent with a similar observation in patients with rheumatoid arthritis of long standing who received histidine supplementation for a completely different reason; the histidine did not improve the rheumatoid arthritis, but invariably significantly improved their low hematocrits [this was a three-clinic study in the eastern United States].

Proposed Course:

With the aid of dietary and metabolic studies and other relevant experimental investigations in uremic patients and rats the contractor will study intestinal absorption of histidine and iron.

Contractor: University of Naples

Amount: $28,000

Title: Studies on Oxystarch in the Treatment of Uremia

Objectives:
To develop a chemical compound which can be administered per os to sequester urea and possibly other uremic toxicants in the gastrointestinal tract, and to develop with the aid of such a compound a suitable adjunctive treatment for patients in chronic renal failure. Also, to explore the feasibility of using oxystarch or related substances as a sorbent of uremic wastes in artificial kidney dialysate solutions.

Major Findings:
A chemically well defined polyaldehyde has been obtained from controlled periodate oxidation of starch. The material does not contain iodine residues and has been proven nontoxic in long-term mouse feeding experiments. The material is stable and non-dialyzable. One hundred grams of "oxystarch" (OXS) bind 33 grams of urea. When given by mouth to uremic patients in divided doses, 20 grams of oxystarch per day result in a daily reduction of 20 milligram percent of blood urea nitrogen. Fecal nitrogen content increases correspondingly and the increased fecal nitrogen can be ascribed to large quantities of urea-derived ammonia irreversibly trapped by oxystarch during passage of the gut. Oxystarch is not hydrolyzed in the intestinal tract, nor absorbed.

The latest experiments have demonstrated that OXS does not chelate food-nitrogen during its passage through the gastrointestinal tract. Controlled experiments in patients receiving OXS who are either fed a Giordano-Giovannetti diet or a regular hospital diet (which contains a higher level of protein) or a protein-free diet have shown that the level of protein intake with the food does not influence the amount of urea/ammonia binding by oxystarch upon passage of the gastrointestinal tract in uremic patients.

Despite the fact that oxystarch is insoluble in the human gut, it is soluble in dialysate solutions, thus it cannot be used as a urea-specific binder in the dialysate of artificial kidneys. However, it has been found that ammonia added in small quantities to oxystarch will change its soluble characteristics; ammoniated oxystarch becomes a water-insoluble material but still retains 60% of its urea-binding activity. Similarly, Dr. Giordana has now shown that oxidized cellulose is an effective and water-insoluble sequestrant.

Proposed Course:
The investigator will test the use of OXS in controlled larger clinical trials for prolonged periods of time in accordance with carefully outlined plans. Also, the contractor will pursue studies with the above mentioned insoluble urea sequestrants in dialysate solutions and in peritoneal dialysis.

CONTRACTOR: Research Foundation (NY)

AMOUNT: $61,898

TITLE: Clinical Efficacy of Oxidized Starch in Uremia

OBJECTIVES: The aim is to evaluate the potential of oxystarch as an adjunct in the therapy of uremia.

MAJOR FINDINGS: Oral administration of oxystarch produces an increase in stool nitrogen as well as a fall in BUN. Oxystarch has proven to be well tolerated and non-toxic.

PROPOSED COURSE: This investigation will continue and extend a two year evaluation of the efficacy of oxidized starch as an intestinal sorbent of potential value in uremia. Patients on maintenance hemodialysis will be placed on oxystarch and dialysis reduced to once a week.

Project Title: National Dialysis Registry

Contract Number: 67-1387

Amount: $124,760 (8 months)

Contractor: Research Triangle Institute

Project Officer: Dr. Keatha K. Krueger

Project Description:

The contractor will continue to accumulate data for the National Dialysis Registry on chronic intermittent dialysis patients, and will provide quarterly statistical printouts of the total registry patients to the Project Officer and cooperating centers. They will furnish the same data on patients in VA centers to the Project Officer.

Data files which link patient or center names with data will be maintained in a manner which insures the maintenance of confidentiality of patient and center data; the manner in which these files are handled have been reviewed and approved.

The National Dialysis Registry has collected data since 1969 from virtually all centers engaged in dialysis in the United States, and prepared from the data statistical analyses for use by the NIAMDD and quarterly reports to all cooperating centers. The data include numbers of patients in center or home dialysis, changes in status of these patients, cause of death in patients that expire, length of time on dialysis, etc. Confidentiality of the center and patient identities is maintained.

Analyses of death by primary cause and multiple decrement life tables are also now available.

CONTRACTOR: University of North Carolina

AMOUNT: $74,296

TITLE: Antithrombogenic Surfaces: Platelet-Interface Reactions

OBJECTIVE: The first area concerns the continuing need for better and more discriminating in vitro and in vivo test models for use in evaluating the blood compatibility characteristics of biomaterials. The second area concerns the composition of the layer of plasma protein which adsorbs to most artificial surfaces which contact blood. The final area, and one which is assuming increasing importance, is the formation of "neointima" upon artificial surfaces and its role in the in vivo compatibility of blood with prosthetic devices.

MAJOR FINDINGS: Dr. Mason's laboratory has used a number of in vitro or ex vivo test systems to estimate the compatibility of a large number of biomedical materials with blood. Starting with a simple test tube method, they have evolved several test systems which measure the activation of the intrinsic blood coagulation system by artificial surfaces. These test systems permit not only an evaluation of some of the coagulation changes which occur in blood exposed to an artificial surface, but also permit the investigation of cellular interactions with these surfaces. Their efforts in this area have lead them to a realization of the importance of the layer of plasma protein which rapidly adsorbs to artificial surfaces upon their exposure to blood. It appears to be within this layer of adsorbed protein that the intrinsic coagulation system is activated and it is to this layer of adsorbed protein that cellular elements adhere. They have studied this layer of adsorbed plasma protein on two previous occasions in attempts to characterize its components. These studies have served to increase their appreciation of the difficulties involved in such work and have stimulated them to look for new approaches to the problem. Finally, they have investigated the possible use of the rhesus monkey as a model for in vivo thrombus generation.

PROPOSED COURSE: Work is proposed in six different areas.

1. One work area will deal with in vitro evaluation of the blood compatibility of dialysis membranes.

2. Continued investigation of the components of the layer of plasma protein which adsorbs to dialysis membranes and other foreign surfaces.

3. The adhesion of platelets to some artificial surfaces found in hemodialysis units will be quantitated.

4. An investigation of possible activation of the intrinsic coagulation system on platelets by artificial surfaces found in hemodialysis units will be carried out.

5. A comparison of six different antithrombotic agents will be made in the captured vortex device model of the rhesus monkey.

6. Limited studies on endothelial cells will be continued.

CONTRACTOR: North Star Research and Development Industry

AMOUNT: $94,962

TITLE: Development of a New Concept in Membrane Structure for Application in Hemodialysis.

OBJECTIVE: The objective of this contract is the development of ultrathin dialysis membranes with performance characteristics superior to those of presently available membranes.

MAJOR FINDINGS: Work on this program recently involved the successful design, assembly, and operation of a basically inexpensive pilot process for continuous casting of double layer ultrathin membranes. Ultrathin cellulose membranes derived from cellulose acetate were fabricated by this process and were shown to possess excellent middle molecule permeabilities. Problems of membrane leakage and high ultrafiltration rates in Kiil assemblies were encountered that delayed initiation of planned clinical trials, but these problems have been resolved in recent months.

PROPOSED COURSE: Five parts are proposed. The most important part will involve the testing of the adequacy of dialysis with the new membrane over six month time periods. Secondly, the mass transport rates of various molecular species during human hemodialysis will be examined to give some measure of small and middle molecule transfer rates. Third, a study of ultrafiltration characteristics of the membrane during routine hemodialysis will be performed. A lesser effort will be devoted to a fourth task involving a study of platelet adhesion to the membrane during routine human hemodialysis. A final task involves a statistical analysis and summary of the data derived from these tests.

CONTRACTOR: Northwest Kidney Center

AMOUNT: $54,764

TITLE: A Comparison of Home Hemodialysis and Home Peritoneal Dialysis in Patients Trained at a Community Dialysis Center.

OBJECTIVES: This study will compare the overall medical effectiveness of home treatment by peritoneal dialysis with that of home hemodialysis in similar patients.

MAJOR FINDINGS: The study is in a preliminary phase and no conclusions can be drawn at this time.

PROPOSED COURSE: The study will compare medical effectiveness, incidence of complications, degree of social and vocational rehabilitation, and cost of the 2 forms of treatment.

CONTRACTOR: University of Pennsylvania

AMOUNT: $140,000

TITLE: Hemodiafiltration

OBJECTIVE: The objective has been the development of an ultrafilter and fluid cycling equipment that will remove molecules of intermediate molecular weight (300-5000 mol.wt.) with a high degree of efficiency.

MAJOR FINDINGS: Amicon has developed a standardized manufacturing technique for 1.6 M^2 hollow fiber ultrafilters that are non-pyrogenic and have a low leak rate. Diafiltration with this unit was compared with dialysis (1.45 M^2 coil) and shown to be as effective in removing urea and creatinine but more effective for the removal of uric acid and phosphate. Inulin clearance for the diafilter was (117 ml/min) quite comparable to the human kidney. Clinical evaluation of 1.6 M^2 ultrafilters on an intermittent basis in an informed anephric volunteer patient was conducted at the University of Pennsylvania and no untoward clinical or biochemical results were noted.

PROPOSED COURSE: Satisfactory completion of six months of hemodiafiltration will demonstrate that this technique is not associated with any acute toxicity from either exposure to a new membrane, a new technique or the loss of "vital solutes" due to the high efficiency removal of compounds with molecular weight of up to 5200. The study protocol by two patients will justify increasing the number of patients treated and the length of their exposure.

The second patient on the six-month crossover protocol will be held on hemodiafiltration for a second six-month period after he completes the one year crossover protocol. This will permit evaluation of chronic exposure to the new technique. Difference in clinical response and further evaluation by a panel of special studies will be completed.

In addition, three other volunteer patients will be started on the one-year protocol. At least one of these patients will be drawn from the maintenance dialysis population with a demonstrated complication (e.g. osteodystrophy with metastatic calcifications, severe high renin hypertension unable to be satisfactorily controlled with salt and water removal, severe muscle wasting and fatigue associated with ascites or peripheral neuropathy developing on 18 hour per week 1.45 M^2 coil program). Each of these patients will be followed using their current clinical protocol. The impact of hemodiafiltration on a maintenance hemodialysis patient who is doing poorly will be evaluated as well as extending experience with the technique in the uncomplicated case.

Contractor: University of Pisa

Amount: $23,436

Title: Albumin, Urea and Insulin Metabolism in Chronic Uremia

Objectives:
To determine the turnover and biochemical aberrations of albumin, urea and insulin metabolism in uremia.

Major Findings:
Sophisticated determination of albumin catabolism through a new triple tracer technique has shown that the intravascular albumin mass is normal in patients on the low, selected protein (Giordano-Giovannetti) diet (even in patients maintained on it up to five years). The extracellular albumin mass, total albumin mass and extracellular to intravascular ratio is reduced in both short-term and long-term patients. The total intravascular albumin mass is preserved within a normal or near normal range. Albumin metabolism is normal in controls and long- or short-term patients. The diet is not directly responsible for the observed albumin depletion since albumin synthesis continues at a normal rate in all cases. Apparently toxic factors of the uremic state itself are responsible.

With regard to insulin it was found that catabolism of insulin was slowed down considerably in uremic patients -- apparently because normally functioning renal tissue is required for a normal rapid breakdown of insulin in the body. It was also found, however, that the rate of secretion of insulin from the pancreas is reduced in uremia -- apparently because of an as yet unknown toxic suppression of normal insulin synthesis in the pancreas or secretion from the beta cells. For uremic patients, the fractional catabolic rate, metabolic clearance and insulin delivery rate were markedly reduced before start of dialysis treatment. After three months of chronic dialysis all three values were decidedly improved and approaching normal values.

Proposed Course:
Continue to evaluate albumin metabolism in patients who are uremic and on a low protein diet, and study albumin, insulin and growth hormone turnover and distribution in uremic patients who are on or off hemodialytic therapy.

CONTRACTOR: University of Rochester School of Medicine

AMOUNT: $79,555

TITLE: Prospective Study of Cytomegalovirus and Herpes Simplex Virus Infection of Patients Undergoing Chronic Hemodialysis.

OBJECTIVE: In an effort to understand the etiology of fever, pneumonia, persistent pericarditis and gastrointestinal problems in hemodialysis patients, they have undertaken a prospective study of cytomegalo virus (CMV) and herpes simplex virus (HSV) infection and antibody response to these viruses.

MAJOR FINDINGS: They have followed several patients from hemodialysis through renal transplant and subsequent to renal transplantation. Their pre-transplant antibody status has predicted post-transplant virus shedding. Only herpes simplex antibody positive patients shed herpes simplex and all CMV antibody positive subjects shed CMV. However, other patients without CMV antibody also shed CMV post-transplant. They did demonstrate that post-transplant CMV shedders with pre-transplant antibody to HSV almost uniformly had CMV viremia and this developed early in the post-transplant period. This contrasted to post-transplant CMV shedders without pre-transplant HSV antibody. If the HSV antibody positive patients did not demonstrate CMV antibody pre-transplant then the CMV virus was associated with high frequency of complication.

PROPOSED COURSE: The studies to be performed during the coming year can be summarized as follows:

a. Monitoring of Virus Shedding of New Dialysis Patients.

b. Twice Weekly Monitoring of HSV Antibody Positive Patients for HSV Shedding.

c. Monitoring of Shedding of CMV in CMV Seroconverters v. CMV Positive Individuals with Stable CMV Antibody Using Special Cultural Techniques and Blind Passage.

d. Evaluation of Immune Responsiveness Groups Differentiated under a, b and c With Polyvalent Pneumococcal Vaccine and Conventional and Live Attenuated Influenza Vaccine.

e. Measurement of Cell Mediated Immune Function:
in Group Listed in b to HSV
in Group Listed in c to CMV and
in Group Listed in b and c to Influenza.

f. Assessment of Adequacy of Dialysis by Studying the Immune Responsiveness of Groups of Patients with Renal Failure not on Hemodialysis to Influenza and Pneumococcal Vaccines.

g. Assessment of Development of Illness with Respect to Shedding of Virus and Immune Competence.

CONTRACTOR: Saint Francis Hospital

AMOUNT: $19,808

TITLE: Clinical and Laboratory Evaluation of Coil Reuse

OBJECTIVE: The objective is the evaluation of coil reuse.

MAJOR FINDINGS: Over the past five years they have prospectively studied the influence of coil reuse on several parameters. Shunt infections and shunt revisions have been lower with coil reuse than in those not reusing coils. There has been no significant difference in the transfusion requirements, mortality or incidence of febrile reactions between the two groups. They have critically looked at coil reuse over the past three years in their Limited Care Facility and at present are obtaining an average of 6.8 dialyses per coil on the UF 145 and 4.5 dialyses per coil on the EX03. They have studied the clearance of various lower and middle molecular weight substances at 0, 4, 9 and 14 reuses. The study is complete and the data on the UF 145 has been statistically evaluated. The remaining data will be completed and a final report sent in. With coil reuse there has been a significant reduction in resistance to blood flow. This has been brought about by a reduction in the pressure at the inlet blood compartment. There has been no change in the pressure at the outlet blood compartment. Thus, there is a reduction in the mean transmembrane pressure. There was no significant change in the pressure at the inlet of the dialysate compartment. There was no change in the ultrafiltration capabilities or the clearances of urea, creatinine, uric acid and phosphate. As anticipated there was an increase in the volume of the reused coil. This data would suggest that with reuse the resistance is lowered because of an increase in the size of the blood compartment with the resultant lowering of the inlet and transmembrane pressures. There was a slight increase in the clearance of Raffinose; however, the clearance of Inulin and B_{12} was unchanged with coil reuse. The net effect of all these changes is not significant for clearance of lower or middle molecular weight substances.

Contractor: Southern Research Institute

Amount: $55,000

Title: Activated Carbon Fibers for Use in Artificial Kidney Devices

Objectives:

To develop activated carbon fibers suitable for use in artificial kidney adsorption devices, and to develop activated carbon hemoperfusion cartridges.

Major Findings:

Fibers with carbon loading of 70% have been prepared in wet-spinning facilities and wound into coils. Cartridges containing these coils have been evaluated to determine their capacity to remove uric acid and creatinine from aqueous solutions. The results are very encouraging.

Proposed Course:

To study cellulosic binder materials to compare their fiber-forming characteristics and their permeabilities to uric acid and creatinine. The best combination of carbon and binder material will be evaluated first in vitro and later in vivo with dogs as test animals.

Project Title: Artificial Kidney - Biochemical Evaluations

Contract Number: 66-1132

Amount: $62,556

Contractor: Stanford Research Institute

Project Officer: Dr. Keatha K. Krueger

Project Description:

The objectives of the current proposal are to:

1. Determine the dialysance of amino acids and their nonprotein conjugates, including peptides, during hemodialysis of patients employing new artificial kidney systems.

2. Examine dialysates obtained during hemodialysis with various artificial kidney systems for the presence of proteins of low molecular weight and peptides (1,000 to 10,000 daltons), and relate this information to similar materials in uremic plasma.

3. Attempt to relate removal or retention of small proteins or peptides in this molecular weight range to the clinical response of uremic patients to various modes of hemodialysis.

Application of chromatographic procedures to plasma from normal subjects and patients being maintained by hemodialysis showed that the patients differed from normal before dialysis in exhibiting areas of elution via 280-nm absorption that were due to mixtures of peptides and amino acids. Microbore column assays indicated the presence of peptides of molecular weight of 1,200 to 1,400 daltons. These materials were reduced in quantity by dialysis, the cellulose acetate HFAK being more efficient than the Model 4 Cordis Dow HFAK in the same patient. Studies in a patient not yet undergoing dialysis also showed the presence of a similar substance. Of particular interest was the finding that the quantity of this material was found to have increased approximately twofold when this same patient exhibited a uremic crisis.

CONTRACTOR: Tulane University School of Medicine

AMOUNT: $82,000

TITLE: The Study of Anemia in Patients with Renal Disease

OBJECTIVES: To study postulated factors that play a role in the mechanism of the anemia associated with uremia.

MAJOR FINDINGS: A radioimmunoassay for erythropoietin is being developed. It appears to be sensitive enough to detect as little as 0.025 milliunits of erythropoietin.

An inhibitor of heme synthesis has been demonstrated in the plasma of seven anemic uremic human subjects. It has an apparent molecular weight of 1 - 3,000.

Seventeen uremic patients have been treated with Fluoxymesterone over 19 - 24 months. Transfusion requirements were significantly reduced in all. Side effects of therapy included muscle soreness, increased hemorrhagic tendency, muscle wasting with increased creatinine production.

PROPOSED COURSE: The investigators will further develop their erythropoietin assay. The plasma inhibitor of erythropoietin will be further characterized. In vitro studies will include the responsiveness of uremic marrows to erythropoietin and to selected steroid hormones.

Contractor: University of Texas

Amount: $34,846

Title: Physiological Transport Parameters in Patients in Peritoneal and Hemodialysis

Objectives:

To obtain a fundamental understanding of the intercompartmental mass transfer characteristics of chronic uremic patients, to elucidate concomitant peritoneal and hemodialyzer extraction rate limitations in terms of metabolite molecular weight,and to define optimum peritoneal dosage cycles and hemodialysis schedules.

Major Findings:

A mathematical model with a maximum of five compartments was developed to describe solute transport in hemodialysis. A similarly two compartmental model was developed for peritoneal dialysis. Both models have been preliminarily tested with good results.

Proposed Course:

The intercompartmental mass transfer parameters, compartmental arrangements, volumes, and associated variables will be evaluated in vivo for both models.

Once the model has been verified, dialysis with hemodialyzers of varying clearances will be simulated and the importance of intrabody resistances to mass transfer in the patient-artificial kidney system will be elucidated. Practical limits for dialyzer efficiencies will be determined.

The peritoneal dialysis model will be used to determine the most efficient mode of peritoneal dialysis.

CONTRACTOR: Tulane University

AMOUNT: $21,000

TITLE: New Concepts in Blood Access Devices

MAJOR FINDINGS:

a. A large number of materials in flat sheet form have been tested for whole blood clotting response by the MCCML technique (Multiple Cell Clotting Method of Lindholm.) Additionally, attempts have been made to develop a bilayer flat sheet of Silastic Medical Adhesive, Silicone Type A (good whole blood clotting response but structurally inadequate to be used as the total cannula material) and other materials.

b. An alternative approach to antithrombogenic cannula development is underway by means of permanently incorporating heparin-binding agent into or in the surface of various materials, such that periodic heparin exposure would recharge the materials.

c. Tube forms of several materials are now being readied for thrombogenicity testing in the test loop A-V shunt implanted in the neck of sheep.

d. Cannula configuration change has been designed with the expectation that cannula clotting will be reduced.

e. The coagulation states of patients have been studied for the purpose of defining why some patients have cannula clotting episodes while others have none.

OBJECTIVE: The basic premise of their research approach is that external arteriovenous cannula shunts would be entirely satisfactory for chronic hemodialysis treatment purposed if certain residual problems with the current cannula system were resolved. They have defined these problems by analysis of their long-term clinical experience with cannulas, and have determined that three problems are foremost, namely, 1) shunt clotting, 2) venous stenosis, and 3) tissue erosion.

CONTRACTOR: University of Utah

AMOUNT: $40,000

TITLE: Thyroid Function in Patients with Chronic Renal Failure

OBJECTIVES: The aim is to define thyroid function in uremic patients and in patients treated with dialysis for extended periods.

MAJOR FINDINGS:

1) Serum thyroxine (T_4) and 131 I uptake values tend to be low normal or low

2) The T_3 resin uptake and thyroxine binding globulin capacity are normal

3) Serum triodothyronine (T_3) and thyrotropin (TSH) levels are normal

4) Serum iodide levels are increased and iodide excess is the most likely explanation of the increased incidence of goiter in chronic renal failure.

CONTRACTOR: Vanderbilt University School of Medicine

AMOUNT: $124,516

TITLE: Neurobehavioral and Clinical Responses to Hemodialysis

OBJECTIVES: The aim is to develop objective, quantitative measures of uremic dysfunction of the nervous system in patients with renal failure 1) as indicators of adequacy of dialysis and 2) as tools for investigating the pathogenesis of uremic neurobehavioral impairments.

The investigators have developed several tests at various levels of nervous system function that correlate with the degree of renal failure and improve with dialysis.

PROPOSED COURSE: They propose to compare standard Kiil dialysis with large surface area dialyzers to see if there are any detectable differences in neurobehavioral performance.

CONTRACTOR: Veterans Administration Hospital-Brooklyn

AMOUNT: $17,000

TITLE: Identification of Proteins Interacting at Plasma/Biomaterial Interfaces. Behavior of Specific Proteins at Interfaces in Relation to Artificial Kidney and Chronic Uremia.

OBJECTIVES:

1) To study basic phenomena involved in adsorption of proteins from plasma onto model and actual surfaces, by employing physical, chemical and immunological techniques for identification and description of the adsorbed species.

2) To apply the methods and results of aim 1) in the study of "freshly used coils" after hemodialysis of patients and of plasma samples from patients with chronic renal failure.

MAJOR FINDINGS: Empirical observations made during the past year include the following: 1) platelets will not adhere to a surface unless proteins do; 2) on some solids fibrinogens was required for platelet adherence; 3) on other surfaces, platelets Wbc and even Rbc adhere, not aided by large amounts of protein; 4) on another type of surface neither platelets nor proteins adhered; 5) interactions by heparin varied according to substrate: polybrene, TOMAC, or protomine sulfate; 6) interactions of plasma with pre-adsorbed 7A-gamma globulin, and the superimposed effect of protamine sulfate and later heparin at the interfaces of this reaction were studied; 7) numerous studies of intact plasma upon silicon crystal slices, on anodized tantalum sputtered glass and on glass: with various permutations compared such as prior deposition of: 7S gamma globulin, globulin, fibrinogen, albumin, etc., and 8) effect of heat upon protein film deposition.

During the past year studies of cuprophan from coils were begun. Adsorption of purified proteins was shown and proteins were identified with antisera stained with Coomassie Blue. Initial studies with formalin indicate it may not interfere with protein identification on used and reused coils.

PROPOSED COURSE: Studies of cuprophan from coils will be continued. The major emphasis will be in evaluating the actual adhesion of proteins and platelets to biomaterials used in dialysis of patients. If and when indicated in vivo studies of materials found adherent to the dialysis membranes may be undertaken using immunologic techniques.

CONTRACTOR: University of Washington

AMOUNT: $80,000

TITLE: Cannula Research

OBJECTIVE: The objective is the improvement and development of hemodialysis cannulae.

MAJOR FINDINGS: Three fistula catheter types reinforced in the extravascular portion with a silastic sleeve were designed. The catheters differ only in the reinforcement of the intravascular portion -- one was unreinforced, one was reinforced with a soft silastic laminate, and one was reinforced with a rigid teflon sleeve. The intravascular segment was shortened to a length of 3 cm. or less. Successful flow studies with all three catheter designs showed that only the extravascular reinforcement was required to make the catheter suitable for dialysis.

However, in animal experiments a new problem of vessel erosion and thrombosis was observed with all three catheter designs. This problem was studied in a "fistula simulator" and two possible causes have been identified. These are catheter length and the point of catheter insertion in relation to the fistula.

CONTRACTOR: University of Washington

AMOUNT: $55,418

TITLE: Acid-Base Chemistry and Human Bone

OBJECTIVE: To understand the various features contributing to uremic osteodystrophy and their interrelationships, thereby designing treatment modalities which will delay, improve or prevent the skeletal abnormalities.

MAJOR FINDINGS: The investigator has proposed that bone buffering of an acute acid load is biphasic, the first phase being a rapid equilibration involving the mobilization of Na and CO_3 followed by a phase of skeletal demineralization. Thirteen dogs were infused over a 6 hour period with 0.1 MHcl. Forty percent of the added acid was buffered by ECF HCO_3 change, plasma proteins, and hemoglobin; the remaining 60% was neutralized by mobilization of CO_3 and Na from bone.

The investigator has classified the bone lesions seen in uremic patients into three categories:

1. Mild - increased forming perimeter, decrease neutral perimeter and increased resorbing surface. The ratio of osteoid to mineralized bone is normal.

2. Malacia - marked increase in ostecid, increased forming perimeter, decreased neutral perimeter, normal resorbing surface.

3. Fibrotic - increased forming perimeter, increased resorbing perimeter.

PROPOSED COURSE: The investigator will continue biochemical and histological analyses of all current biopsy material. In addition he will use x-ray diffraction to determine the degree and size of crystallinity and amorphous calcium phosphate. These parameters will be used to follow the effects of $1,25(OH)_2D_3$ on the course of renal osteodystrophy. The role of Mg toxicity in the etiology of renal osteodystrophy will be evaluated in both man and rats.

CONTRACTOR: University of Washington

AMOUNT: $80,955

TITLE: Pharmacokinetic Studies in Chronic Renal Disease and Hemodialysis.

OBJECTIVES: To provide rational drug regimens to be used in patients with varying degrees of renal insufficiency. To provide rapid, reliable drug assays.

MAJOR FINDINGS: Gentamycin studies have revealed:

1. Significant removal of gentamycin occurs during dialysis which cannot be disregarded when treatment is planned.

2. Approximately 50% of the loading dose should be given following each dialysis.

3. The pH of the dialysate appears to effect the dialyzer clearance of gentamicin.

In functionally anephric patients the half-life of ethambutol is prolonged approximately five-fold. The mean dialyzer clearance is 50 ml/min.

PROPOSED COURSE: The investigator plans to continue along the same lines -- drugs to be studied include flucytosine, procainamide, quinidine and azathioprine.

CONTRACTOR: University of Washington

AMOUNT: $175,000

TITLE: Clinical and Engineering Research on Hemodialysis Procedures

OBJECTIVES: The aim is to determine what constitutes minimum dialysis and optimum dialysis

MAJOR FINDINGS: They have developed a mathematical model which attempts to predict the results of manipulation of dialytic technique. Several tests have been designed to provide useful information regarding patient well-being. Preliminary findings suggest that it may be possible to dialyze patients for shorter periods of time without affecting patient well-being.

PROPOSED COURSE: Proposed work is aimed at further development of the mathematical model, and validation of the measures of patient well being, so as to allow better assessment of minimum and optimum dialysis.

Contractor: University of Washington

Amount: $50,000

Title: Development of a Home Peritoneal Dialysis System

Major Findings:

Through several years of contract support a compact, portable, peritoneal dialysis system has been developed. It prepares sterile dialysis solution from tap water and a sterile dialysate concentrate at the patient's bedside, and automatically administers dialysis while the patient rests or sleeps. Following laboratory testing and preliminary clinical testing the project is entering into more extensive and long-term clinical testing.

Proposed Course:

Continue clinical evaluation of the peritoneal dialysis system. This evaluation will include bacteriological, mechanical, electronic and electric safety and reliability of the equipment itself, and also the evaluation of device-patient interaction and treatment effectiveness.

Contractor: Washington University

Amount: $28,350

TITLE: Removal of Uremic Waste Metabolites From the Intestinal Tract by Microencapsulated Reactants.

OBJECTIVES: To develop a new technique for removing waste metabolites from the gastrointestinal tract by encapsulating specific reactants inside hollow polymeric spheres.

MAJOR FINDINGS: Aluminum hydroxide gels were generated under conditions which optimize anion capacity. These gels show *in vitro* phosphate binding capacities nearly twice that of the best commercial antacid gels. Dialdehyde starch and polyacrolein show ammonia binding capacities near 10 wt.%, equivalent to nearly 18 wt.% urea removal.

PROPOSED COURSE: This project has been terminated.

CONTRACTOR: Washington University School of Medicine

AMOUNT: $56,760

TITLE: Bone Metabolism in the Chronic Uremic State and its Response to Vitamin D and Diphosphonate Therapy

OBJECTIVE: To study in-depth the effects of 25OH-D_3 and/or dichloromethylene diphosphonate (Cl_2MDP) on skeletal maturation during the course of chronic renal insufficiency.

MAJOR FINDINGS: A defect in bone collagen and crystal maturation has been documented in the uremic state. Treatment with either $25OHD_3$ or Cl_2MDP prevented the changes in collagen maturation. Combined therapy caused a greater improvement than either agent alone. The improvement seen with therapy can be correlated with a decrease in turnover as evidenced by decreased urinary excretion of Ca, Pi and hydroxyprotine.

Cl_2MDP did not alter plasma 25OH-D_3 levels.

PROPOSED COURSE: The investigator plans to continue along the same lines beginning therapy during a more advanced stage of uremia. In addition he plans to better define the collagen maturational defect in terms of the types and nature of inter-fiber cross links.

Contractor: International Rice Research Institute (PH-43-67-726)...
U.S.-Japan Program

Amount: $40,165

Title: Improvement of the Protein Content of Rice

Objectives:

The primary objective of this project is to determine the feasibility of incorporating a high level protein into commercially produced rice in Southeast Asia. Presently used commercial varieties of rice contain from seven to seven and one-half percent protein. The biological quality of rice protein is fairly high and an increase in the protein level in rice would go far toward alleviating protein undernutrition in countries for whom rice is the mainstay of subsistence. Since 60 percent of the world's population subsists primarily on rice, the implications for an amelioration of the chronic protein shortage in many underdeveloped countries, should this project prove successful, are quite obvious. If the present protein level of rice could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in major areas throughout the world.

Methods Employed:

The modus operandi involves the screening for high protein content, of over 7,000 varieties of rice archived in the International Rice Research Institute collection, followed by selective breeding and development of a commercially feasible variety which incorporates an improved protein level together with all the necessary attributes which would make this rice attractive to the average Southeast Asian rice grower, namely high yield per acre, resistance to endemic diseases, strong response to fertilization, and -- most important -- desirable organoleptic qualities with respect to stickiness, mouth-feel and taste.

Major Findings:

Over 100 rice varieties have been identified among the many thousands tested which contain between 13-1/2 to 17-1/2 percent protein. Thus far six high protein strains of rice (some from Hungary and some from Southeast Asia) have been successfully crossbred with a new rice variety (IR8) which has been developed previously at the International Rice Research Institute and which possesses near-ideal plant characteristics including disease resistance and a very high yield per acre. Some of the new hybrid lines have shown as high a protein content as the high-protein parent. Others, of fairly acceptable "plant type" have a protein content of +-11 percent, i.e., contain 50 percent more protein than currently produced commercial rice. Based on the most recent breeding work involving progeny of successful crosses between the six high-protein strains and IR8 the project staff has shown that the protein content of rice is indeed a genetically determined characteristic. Protein efficiency ratio (PER) studies, using rats, of several parent rice strains have been carried out by Dr. Bressani at the Institute of Nutrition of Central America and Panama (INCAP) and future PER studies of the new hybrid varieties are contemplated. Dr. Helen Clark of Purdue University has demonstrated in nitrogen balance studies involving students that the experimental high-protein rice which contained 1.8 times as much protein as Bluebonnet rice (a commercially controlled rice containing the conventional 7 percent protein) caused significantly higher nitrogen retention ($P < 0.01$), which is attributed to the increased amounts

of essential amino acids. The respective mean positive balances were 1.41 = 0.89g and 0.24 = 0.31g. Addition of supplementary nitrogen to make the Bluebonnet rice isonitrogenous with the BPI-76-1 variety did not cause a statistically significant improvement. All subjects were in negative balance when 320g Bluebonnet rice were made isonitrogenous with 480g of the same rice by adding nonspecific nitrogen.

In nitrogen balance studies in toddlers (1.5-2 years old) with C. L. Intengan (Food and Nutrition Research Center, Manila), three milled rice samples with 7.7, 10.3, and 11.9 percent protein (N x 6.25) were used in a diet which had a constant rice-to-fish ratio of 100:17 (wt/wt). The fish fillet (surgeon, Acanthurus bleakeri) had 20 percent protein and 10 percent lysine in its protein. Results so far indicate that composite diets with higher rice protein content had lower chemical score but resulted in higher nitrogen since percentage nitrogen absorption and retention were maintained (Table 4). In earlier trials in which IR480-5-9 milled rice with 11.9 percent protein was used as the sole source of protein (mean daily intake of 314 mg N/Kg body wt) for four children, nitrogen absorption was 75 percent and nitrogen retention was 37 percent of intake.

Also improved lines have been developed with a protein content and yield potential comparable to IR480-5-9 and carying resistence to blast, bacterial blight, green leafhopper and brown planthopper (Table 1). Resistance to tungro and grassy stunt virus diseases is being incorporated into these lines. Lines with PTB 18 and Oryza nivara as parents continued to give promising results (Table 1 and 2). Lines from PTB 18, the most promising of which is IR1702-158-3 (Table 1) carry resistance to tungro, green leafhopper and brown planthopper.

Significance:

(Please see above under Objectives.) Ordinary commercial rice does not contain enough protein to support normal growth and development of infants and children maintained on a predominant rice diet, and does not protect the adult population from serious protein under-nutrition. Rice is the principal food of 60 percent of mankind. If the present protein level of rice (approximately 7-1/2 percent) could be elevated by several percentage points an automatic and sweeping nutritional improvement would take place in all the populations for whom this one crop represents the nutritional mainstay. Such a development would concomitantly relieve the current burden of the United States which supplies hundreds of millions of dollars' worth of supplementary foods to underdeveloped rice-eating countries. The potential nutritional improvement among Southeast Asian populations and underdeveloped rice-eating countries throughout the world is an important aim of the U.S.-Japan Program and of the applied nutrition program of the Institute.

Proposed Course:

At this point and time because of a change in the guidelines of the U.S.-Japan Program, the agricultural portion (breeding experiments) of this can no longer be supported by NIH funds and we have been successful in having the International Rice Research Institute accept responsibility for this funding. The human nutrition aspects of this very promising project will continue to be pursued by NIAMDD. Feeding trials with toddlers with

rice: legume mixtures will continue. Amino acid analysis of rice samples and the diets from the study of Dr. Intengan will be done at IRRI.

A related nitrogen balance study in pre-school children is being negotiated with Dr. Sheila Pereira (Christian Medical College, Vellore, Tamil Nadu, India) using rice: legume diet (e.g., black gram dhal, (Phaseolus mungo). Diets in this region of India are characterized by having about 80 percent of calories and protein from milled rice.

In the past year work has continued to focus on various aspects of diabetes mellitus and arthritis in the American Indian, and on the identity of goitrogenic activity produced by Esherichia coli.

Dr. Peter H. Bennett was elected to the Fellowship of the Faculty of Community Medicine of the Royal Colleges of Physicians of the United Kingdom. He was also elected President of the Society for Epidemiologic Research for 1973-74.

SOUTHWESTERN FIELD STUDIES SECTION

Dr. Stephen Kamenetzky, formerly staff associate, received the 1973 Post-doctoral prize of the Society for Epidemiologic Research for work performed on Diabetic Nephropathy in the Pima Indians.

Dr. Stephen E. Dippe, Associate Chief of the Section, left the Section on June 1st. His departure will place an increased workload on other members of the Section, until a replacement is recruited.

Prediabetes

A study of reputed abnormalities associated with the prediabetic state has been launched. Genetic prediabetic Pima Indian subjects (the normal offspring of two diabetic parents) are being compared with the normal offspring of normal Pima Indian parents, and with age-sex-weight matched normal, non-Indian controls. The tests performed include insulin and glucose determinations during oral, intravenous and cortisone glucose tolerance tests, and arginine infusion tests. Muscle capillary basement membrane thickness is being measured by both Siperstein and Williamson in each of these groups and also in newly diagnosed and long term Pima Indian diabetics with and without the specific vascular complications of diabetes.

This study should resolve a number of major issues in diabetes research, namely whether or not biochemical and/or capillary vascular abnormalities can be detected in the subject who is genetically predisposed to develop diabetes.

Long term follow-up of these Pima Indian subjects will also elucidate whether or not the reputed abnormalities of "genetic" prediabetes are in fact predictive of diabetes and whether or not the "true" prediabetic state differs from the "genetic" prediabetic state.

Insulin Levels in Prediabetes and Diabetes

Insulin levels during oral glucose tolerance tests in "true" prediabetic subjects have been investigated by comparing insulin secretion in subjects with normal glucose tolerance tests who subsequently developed diabetes over a 4 year period with those who did not. No significant differences in insulin levels between the true prediabetics and the non-diabetic subjects were found.

The characteristics of insulin secretion in response to an oral carbohydrate load over a wide range of glucose tolerance has also been investigated in the Pima Indians. As in other populations absolute insulin deficiency is characteristic of severe diabetes. In subjects with intermediate degrees of glucose tolerance wide variation in insulin levels was found with high mean insulin levels. These data appear to substantially resolve the controversy concerning whether hypo- or hyperinsulinemia is characteristic of "mild" diabetes.

Coxsackie B_4 Infection and Diabetes

The hypothesis that Coxsackie B_4 virus infection may cause or precipitate diabetes mellitus has been examined among the inhabitants of the Pribilof Islands. Five years ago a well documented epidemic of Coxsackie B_4 infection occurred in a previously virgin population. Below 20 years of age, where the attack rate was very high, not a single case of diabetes was found.

In the adults the incidence of diabetes in those with Coxsackie B_4 infection was similar to that among that who were not infected. The data provide a strong basis for concluding that Coxsackie B_4 is not a common cause or precipitant of diabetes in the Aleut, and that it is unlikely to be so in man in general.

Glucose Tolerance and Vascular Complications of Diabetes

The relationship of fasting glucose levels to the previously reported bimodal frequency distributions of one- and two-hour post-load glucose levels has been investigated. Subjects with glucose levels in the first component of these curves, which represents non-diabetic subjects, have fasting plasma glucose levels of less than 115mg/100ml, levels which are universally agreed to be normal. Subjects in the second mode characteristically have fasting glucose levels above 115mg/100ml, and this level rises rapidly and proportionately with the post-load levels. These findings substantiate the significance of the previously demonstrated bimodality.

The significance of the bimodality is further substantiated by the observation that multiple complications of diabetes are found exclusively and in high frequency in subjects who fall in the second component of the glucose distributions. There is no indication that persons in the first component of the glucose distribution even at the higher glucose levels, are prone to develop the multiple specific complications of diabetes mellitus.

Extensive data on serum lipid levels in Pima Indians are being collected. Baseline data are available on serum triglyceride levels and cholesterol levels. It is hoped that these data will help elucidate the extraordinarily low prevalence of coronary heart disease observed in the Pima population as a whole, and among the diabetics in particular. The relationship of serum lipids to the incidence of the specific complications of diabetes will also be determined.

The high prevalence of obesity among the Pima Indians may be related to the high prevalence of diabetes, since the incidence of glucose intolerance is greater in the obese, especially in the 25-44 year age range. An understanding of the patterns of development of obesity in this population is being sought. Weight gain seems to be relatively normal in children up to 5 years of age, but then accelerated weight gain may be important as an initial lesion which ultimately leads to diabetes mellitus many years later.

The prevalence of diabetes mellitus in the San Carlos Apache Indians, reported here in preliminary form, will provide important information on the relative contribution of environment and genetics to the prevalence of diabetes mellitus in the Southwestern Indian and will complete a long range project defining the geography of diabetes in the Southwestern American Indian.

Arthritis

This year data collection has been completed on the ten-year follow-up of Pima Indians initially examined in 1963. These data will enable analysis of the prognostic significance of rheumatoid factor and other variables and enable a more detailed description of the natural history of the development of rheumatoid arthritis than has previously been possible within a natural population.

METABOLIC DISEASE EPIDEMIOLOGY UNIT

Partial purification of material with goitrogenic activity from both cultures of Esherichia coli has been achieved, and in vitro methods of assay are being devised. It is hoped that characterization and identity of this material will be achieved soon.

Serial No. NIAMDD-EFS-1(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Prevalence of Diabetes in Indian Populations in the Southwestern United States

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Stephen E. Dippe
Mary Gieche

Cooperating Units: Indian Health Service (PHS)

Man Years:

Total:	1.0
Professional:	0.3
Others:	0.7

Project Description:

Objectives:

1. To determine the prevalence of diabetes among various native populations in the Southwestern United States.

2. To formulate and test hypotheses which may explain the high frequency of diabetes mellitus among some of these populations.

Methods:

Random samples of total populations will be examined by means of standardized tests of glucose tolerance to enable the prevalence of glucose intolerance to be determined. Other examinations will be performed to document the frequency of other disorders when appropriate.

Major Findings:

A survey is being conducted to determine the frequency of glucose intolerance among the San Carlos Apache Indians aged 35 years and over residing on the San Carlos Apache Indian reservation in Southeastern Arizona.

To the present time a 75gm oral carbohydrate tolerance test have been performed on approximately 400 persons, and will be continued until a high proportion of the adult population has been examined.

Preliminary results indicate that the prevalence of hyperglycemia is higher among this group than among the closely related, but geographically separated White Mountain Apache.

Some 24 per cent of the San Carlos Apache so far examined have hyperglycemia or previously known diabetes mellitus compared to about 11 per cent among the White Mountain Apache.

Significance of Research:

These and previously reported findings suggest that the prevalence of diabetes in populations may be environmentally determined as well as being related to the genetic origins of the populations.

Proposed Course:

Upon completion of the San Carlos survey analysis of these data and those gathered in other Indian groups in the Southwestern United States will be analyzed to determine the environmental and genetic correlation of diabetes among American Indian populations in this region.

Honors and Awards:

None

Publications:

None

Serial No. NIAMDD-EFS-2(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Prospective Study of the Natural History of Diabetes Mellitus in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Stephen E. Dippe
Dr. Peter J. Savage
Dr. Richard F. Hamman
Dr. Stephen L. Aronoff
Dr. Max Miller
Dr. Arthur G. Steinberg
Dr. Norman B. Rushforth
Dr. Elmer J. Ballantine
Dr. Irving M. Liebow
Dr. Frederick A. Rose
Dr. John Turner

Cooperating Units:
Indian Health Service
Case Western Reserve University
National Eye Institute
University of California, San Diego

Man Years:

Total:	6.6
Professional:	2.4
Others:	4.2

Project Description:

Objectives:

1. Determine the prevalence and incidence of diabetes mellitus among those five years of age and over on the Gila River Indian Reservation.

2. Evaluate factors associated with the progression from pre-diabetic state to subclinical diabetes mellitus and to clinical diabetes.

3. Study the association between diabetes and rate of appearance and progression of vascular complications.

4. Investigate influence of tribe, age obesity, parity, and other factors on changes in glucose tolerance and their relationship to diabetes mellitus.

Methods:

1. The following tests and examinations will be repeated at two yearly intervals on all Indians five years of age or more living on the Sacaton Service Unit portion of the Gila River Reservation.

a. Clinical interview
b. Physical examination
c. Modified glucose tolerance test

and in addition on those aged 15 years and over

d. Fundus photographs
e. Electrocardiogram
f. Tests for vibratory sensation threshold
g. Soft tissue radiographs for vascular calcification

2. Some of the above tests and examinations will also be done on Indians of other tribes or living under different environmental conditions for comparison.

Major Findings:

1. Serum Immunoreactive Insulin: Since the development of the radioimmunoassay for measuring insulin, controversies exist concerning insulin levels in the mildly carbohydrate intolerant individuals. A major concern has been whether or not delayed insulin release is an early lesion in diabetes.

Serum insulin levels were measured in 396 Pima Indians at time 0, 30, 60, and 120 minutes following a 75gm oral carbohydrate load. The sample represents a spectrum of glucose tolerance and intolerance ranging from a two hour plasma glucose of 61 to 724mg/100ml. Bimodality was noted in the one half, one hour and two hour plasma glucoses. There was also suggestion of bimodality in the fasting glucose distribution. The insulins taken at the similar time periods demonstrated no evidence of bimodality but had a log-gaussian distribution.

In the clearly normal range, two hour plasma glucose less than 120mg/100ml, peak insulin occurred at one hour. In those who had a two hour plasma glucose from 120 to 169mg/100ml, serum immunoreactive insulin was higher at all time points than the clearly normal individuals. There was no evidence of delayed insulin release. In the moderate carbohydrate intolerant group, two hour plasma glucose of 170 to 239mg/100ml, two hour insulins were clearly higher than the normal group and the half hour was less than the normal group. However, at all time periods the insulin levels in the moderately carbohydrate intolerant group were lower than the mildly carbohydrate intolerant group. This indicates that insulin was insufficient at all time periods and that the two hour insulin level was higher because of the greater glucose stimulus.

2 hr Pl Glucose Range mg/100ml	No.	Mean Plasma Glucose (±SEM) mg/100ml			
		Fast	1/2 hour	1 hour	2 hour
120	49	89±2	138±5	133±5	101±2
120-169	91	97±2	157±3	173±4	144±2
170-239	77	108±2	178±3	217±4	199±2
240-399	116	162±5	252±6	307±6	315±4
400	62	270±8	365±9	442±9	467±7

2 hr Pl. Glucose Range mg/100ml	No.	Mean Serum Immunoreactive Insulin (±SEM) μU/ml			
		Fast	1/2 hour	1 hour	2 hour
120	49	30±2	132±12	155±14	139±20
120-169	91	45±7	153±18	187±21	223±20
170-239	77	41±3	100± 6	145±15	178±15
240-399	116	41±2	71± 4	88± 6	99± 6
400	62	32±3	39± 3	41± 4	40± 3

No significant differences were found in the various age groups or between sexes. The obese had higher insulin values than their less obese counterparts at each of the two hour plasma glucose levels. The pattern of distribution of the insulin values was essentially the same as in the less obese group but insulin values were higher.

2. Insulin levels in prediabetes: Serum immunoreactive insulin levels were analyzed on individuals who demonstrated clear decompensation of glucose tolerance within a four year period. Thirteen individuals were identified whose two hour plasma glucose changed from less than 160mg/100ml to greater than 275mg/100ml. These subjects were matched to controls in whom insulin levels were available and in whom the two hour plasma glucose remained less than 160mg/100ml. The insulin levels prior to the development of marked carbohydrate intolerance were no different than in the controls. The mean two hour plasma glucose level in the decompensated was 375±28mg/100ml. There was no significant difference in any of the time periods in insulin level between the normal and prediabetic subjects.

3. The relationship of Fasting and Two hour Glucose levels: Bimodal frequency distributions of two-hour plasma glucose levels have been previously reported with a two-hour plasma glucose level of around 240mg/100ml at the antimode. We postulated that the first mode represented the non-diabetic population and the second mode the diabetic population.

In order to examine the association of fasting glucose homeostasis with the glucose levels following an oral glucose load, a study was conducted in 680 Pima Indians in whom a full glucose tolerance test was performed. The subjects were grouped according to two-hour plasma glucose level which ranged from less than 100mg/100ml up to greater than 480mg/100ml.

From the group with two-hour plasma glucose levels less than 100mg/100ml (mean = 86.8) to the group with two-hour plasma glucose 225-244mg/100ml (mean = 235.3) the mean fasting glucose rose linearly from 86.8 ±2.4mg/100ml to 114.1 ±4.9mg/100ml. For every 10mg rise in the two-hour plasma glucose there was only a 1.9mg rise in the fasting glucose level. In groups with higher two-hour plasma glucoses the fasting glucose again rose linearly, but four times as steeply as those with better tolerance. For those with two-hour plasma glucose 265-248mg/100ml (mean = 273.0) to the group with the two-hour plasma glucose of 480mg/100ml and over (mean = 524.9) the mean fasting glucose rose from 127.6 + 3.6mg/100ml to 315.4±11.3mg/100ml. In this range for each 10 milligram rise in the two-hour plasma glucose there was a corresponding 7.6mg rise in the fasting glucose level. The data indicate that two separate associations exist between the fasting and two-hour plasma glucose. In those with the two-hour plasma glucose less than approximately 250mg/100ml the fasting glucose rose slightly from 80 to 115mg/100ml. Those with higher glucose levels had definite elevation in the fasting glucose level and would be considered to have diabetes by all accepted criteria.

From these data it was concluded that indeed the first mode represents those who have normal fasting glucose homeostasis and the second mode is composed of those who have diabetes with a deficiency in fasting glucose homeostasis.

Relationship of Fasting 1/2 hour and 1 hour Glucose to 2 hour Glucose Levels

2-hr. Pl. Glucose Range	No.	Mean Plasma Glucose (mg/100ml) Fasting	1/2 hr.	1 hr.	2 hr.
<100	31	87	131	122	87
100-114	39	93	144	137	107
115-129	39	90	144	156	122
130-144	41	98	161	169	138
145-159	45	97	160	182	152
160-174	29	103	166	188	167
175-189	42	105	169	209	181
190-204	40	105	172	214	196
205-224	34	109	183	231	214
225-244	35	114	192	234	235
245-264	33	132	215	256	254
265-284	30	128	215	269	273
285-304	19	139	220	274	297
305-329	34	163	242	312	316
330-354	22	184	283	350	341
355-379	30	202	285	342	366
380-404	22	200	298	364	392
405-429	26	230	320	398	416
430-454	26	243	326	405	441
455-479	28	259	361	428	468
≥480	35	315	413	499	525

4. Serum Triglyceride Levels and their Relationship to Glucose Tolerance: Serum Triglyceride levels were measured in 579 Pima Indians. There was a gradual rise in the mean fasting plasma glucose, two hour plasma glucose and serum cholesterol level in persons with serum triglyceride level from less than 50mg/100ml to 199mg/100ml. In the triglyceride range of 200-299mg/100ml there was little change in fasting glucose or two hour glucose level. Serum cholesterol also changed little. The mean two hour plasma glucose was above 400mg/100ml when the serum triglyceride level was above 300mg/100ml.

Triglyceride Levels
mg/100ml

		<50	50 to 74	75 to 99	100 to 125	125 to 149	150 to 174	175 to 199	200 to 224	225 to 249	250 to 274	275 to 299	300 to 349	350 to 399	400 +
Fast.Pl. Glucose mg/100ml	N	12	46	95	90	101	55	45	32	31	22	8	15	12	15
	X	102	116	122	129	144	159	163	156	171	176	157	189	252	228
	S.E	12	7	5	6	7	10	11	13	15	15	36	22	37	24
2hr.Pl. Glucose mg/100ml	N	11	47	95	89	99	55	45	33	31	22	8	15	12	15
	X	152	211	226	230	263	279	292	277	289	321	273	332	393	347
	S.E	30	15	12	12	12	16	17	21	25	24	59	37	47	36
Serum Cholest. mg/100ml	N	12	47	95	90	101	55	45	33	31	22	8	15	12	15
	X	135	156	163	174	187	189	195	196	194	197	226	209	216	216
	S.E	8	4	3	3	4	5	5	5	6	9	13	7	11	10

5. Arterial Calcification, Retinopathy, and Proteinuria: We have previously found a strong association of elevated plasma glucose to arterial calcification, retinopathy, and proteinuria. Analysis was performed looking at all three of these complications in relationship to age, diabetes status, and duration of diabetes. The percent free of any of the three complications was always higher in the females in each of the duration groups for diabetes. In the newly diagnosed diabetic 65% of the males and 77% of the females were free of any of these three complications. With durations of less than 5 years, the frequency dropped to 40% for males and 62% for females. In the 5 to 9 year duration group, it was 33% and 50%, in the 10-14 years 7% and 30%, and 15 years or greater 0% and 20%, respectively.

In those in whom the duration of diabetes was greater than 15 years, three persons could be identified out of a total of 18 who had none of the complications present. Out of 718 Pima Indians aged 25 and older who had a two-hour plasma glucose less than 200mg/100ml, there was only one person who had arterial calcification, retinopathy and proteinuria occurring together. On the other hand, 24 individuals had all three complications present out of 345 persons who had a two-hour plasma glucose greater than 200mg/100ml.

Complications and their relationship to Duration of Diabetes

Duration of Diabetes	Sex	Complications * NONE		P		R		P+R		C		P+C		R+C		P+R+C	
		N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Newly	M	36	65	2	4	0	0	1	2	13	24	1	2	1	2	1	2
Diagnosed	F	56	77	11	15	0	0	1	1	4	5	1	1	0	0	0	0
<5 years	M	18	40	2	4	1	2	0	0	13	29	2	4	5	11	4	9
	F	40	62	2	3	5	8	1	2	11	17	0	0	5	8	1	2
5-9	M	10	33	3	10	4	13	0	0	7	23	0	0	3	10	3	10
years	F	20	50	2	5	2	5	2	5	8	20	1	2	4	10	1	2
10-14	M	1	7	0	0	2	14	0	0	2	14	3	21	3	21	3	21
years	F	7	30	1	4	2	9	1	4	5	22	2	9	0	0	5	22
>15	M	0	0	0	0	1	9	1	9	5	45	1	9	2	18	1	9
years	F	3	20	0	0	1	7	0	0	2	13	0	0	2	13	7	47

* P = proteinuria
R = retinopathy
C = arterial calcification

6. Cholesterol Levels in the Pima Indians: The Pima Indians have previously been reported to have lower levels of serum cholesterol than their Caucasian counterparts. An evaluation was made of the cholesterol levels in the children. A sample of cord blood from 29 newborns showed a level of 86±3mg/100ml for males and 88±2mg/100ml for females. Between birth and five years of age, the level of the serum cholesterol increased. From five years of age to fourteen years of age, the level of cholesterol remained stable with a mean level of 145±1mg/100ml for males and 144±1mg/100ml for females. A rise in cholesterol was observed at about 17 years of age and continued through the early 20s. The mean serum cholesterol level from 15 to 19 years was 152mg/100ml for males and 158mg/100ml for females.

Cholesterol levels at menopause were determined on a large group of Pima Indian females. Out of 65 women who could be clearly identified as having cholesterol level measured just prior to and after menopause there was no indication of elevation of cholesterol levels between the two points. No evidence was found to support the hypothesis that the menopause is associated with an increase in serum cholesterol level. Diabetic women were found to have significantly higher serum cholesterol levels than non-diabetic women in the 34-54 age group. (186±3mg/100ml vs. 176±3mg/100ml)

7. Growth and Development Among Pima Indian Children: The Pima Indians, are more obese than non-Indian populations. The adult Pima Indian has been shown to weigh on the average 150% of his desirable body weight. To establish the pattern of development of obesity, Pima Indian children were examined between the ages of 5-25. The mean weight of a Pima child at age 5 was 21.0 kilograms for males and 20.4 kilograms for females, which is similar to Caucasian norms. Between the ages of 7-11, there is a gradual

and much more rapid weight gain than that found among Caucasian children. By age 11, the average Pima male weighed 47.1 kgs. and the average female 48.4 kgs., which is 28% above the respective Caucasian means. During this very striking increase in weight during pre-pubertal years was not associated with an increase in the mean two-hour plasma glucose.

Weight gain from 12 to 18 years in Pimas was similar to that of Caucasians. From age 18 to 25, the weight of the Pima once again increased. At age 25 males weighed 91±3kg., females weighed 83±2kg. These weights are 28% and 37% above mean weight for Caucasian adult. During this period mean 2 hour plasma glucose increased from 103 to 140mg/100ml.

Significance of Research:

The relationship between serum insulin levels and glucose tolerance have shown that much of the controversy concerning the characteristics of insulin secretion in mild carbohydrate intolerance has resulted from the wide variation in levels and the fact that the mean insulin levels vary considerably over a narrow range of glucose tolerance.

The question of whether abnormalities in insulin secretion preceed the onset of unequivocal glucose intolerance has been examined and no differences in insulin response to an oral glucose load were found in true prediabetic subjects, suggesting that islet cell dysfunction may not be present prior to the onset of glucose intolerance. This suggests that other lesions such as increased peripheral resistance may well be the precipitating factor.

Since obesity is a factor known to change peripheral resistance to insulin a more detailed analysis of its development and significance is required. Preliminary results indicate that the development of obesity among Pima Indian children starts at about the age of 6 years, and from age 11 years onwards. Pima Indians are approximately 30 percent heavier than Caucasians.

The glucose response to an oral carbohydrate load and the fasting glucose level has been shown to be closely related. Persons falling into the diabetic component of the frequency distribution of one and two hour post load glucose levels characteristically have fasting glucose levels above the normal range. The significance of this fact is enhanced by the observation that the multiple complications of diabetes mellitus were found almost exclusively among subjects who fell into the hyperglycemic component of the frequency distribution curves, and by implication, among those with abnormally high fasting glucose levels.

The role of serum lipid levels in determining the lack of coronary heart disease in the Pima Indian population was further explored. Serum triglyceride levels were shown to be high among those with glucose intolerance, whereas serum cholesterol levels increase during childhood and adolescence, but remain low in adults and do not show any significant rise attributable to menopause in females.

Proposed Course:

The long term prospective studies of diabetes and its vascular complications will be continued. Since many subjects have now had documented glucose intolerance for ten years, detailed analyses of the factors related to the incidence, rather than prevalence, of the specific complications will be initiated. Such analyses offer a much greater likelihood for identification of factors of causal significance than is possible with prevalence data alone.

Further studies to characterize factors related to the onset of glucose intolerance will also be undertaken and will include determination of the relationship of insulin secretion and obesity to the evaluation of glucose intolerance.

Honors and Awards:

Dr. Stephen A. Kamenetzky received the 1973 Post-doctoral prize of the Society for Epidemiologic Research for his work on Diabetic Nephropathy in the Pima Indians.

Publications:

Kamenetzky, S.A., Bennett, P.H., Dippe, S.E., Miller, M., and LeCompte, P.M.: A Clinical and Histologic Study of Diabetic Nephropathy in the Pima Indians. Diabetes 22:61-68, 1974.

Serial No. NIAMDD-EFS-3(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Prospective Study of the Natural History of Arthritis and Rheumatism in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Thomas A. Burch
Dr. Stephen E. Dippe
Dr. Peter J. Savage
Dr. Richard F. Hamman
Dr. Stephen L. Aronoff

Cooperating Units:
Indian Health Service (PHS)
Hawaii State Health Department

Man Years:

Total:	1.2
Professional:	0.5
Others:	0.7

Project Description:

Objectives:

1. Determine the progression of clinical, radiological and serological features of joint diseases affecting the Gila River Indians with particular attention to rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and gout.

2. Determine environmental and genetic factors associated with development and progression of arthritis and rheumatism.

3. To compare and contrast the clinical course of rheumatoid arthritis in sero-positive individuals with that of sero-negative arthritics.

4. Determine the predicative and prognostic significance of rheumatoid factor in relation to the clinical course of rheumatoid arthritis.

Methods:

1. The following tests and examinations will be repeated on all Indians five years of age or over, living on the Sacaton Service Unit portion of the Gila River Reservation.

a. Clinical interview
b. Physical examination
c. Radiographs of hands, feet, and cervical spine. Males over age 14 and females over age 44 will have a pelvis film taken.
d. Laboratory determinations: rheumatoid factor, uric acid.

2. The data from the above examinations and tests as well as demographic, pedigree, environmental information, and data on other conditions and diseases will also be collected.

Major Findings:

A large group of Pima Indians without evidence of rheumatoid arthritis, but with serologic evidence of rheumatoid factor, and a group of age-sex-matched controls have been followed at two yearly intervals over the past 10 years. Initially mortality in males with rheumatoid factor was extensive, but over the past eight years mortality in those with and without rheumatoid factor has remained the same. Analysis is now proceeding to determine whether rheumatoid factor preceeds the development of other manifestations of rheumatoid arthritis.

Significance:

This study represents one attempt to define etiologic factors in rheumatoid arthritis. The data will elucidate whether or not subclinical evidence of rheumatoid arthritis may be present for many years prior to the emergence of clinical evidence of arthritis.

Proposed Course:

The natural history of various arthritides will be examined now that 10 year follow up data is available.

Honors and Awards: None

Publications:

Beasley, R.P., Wilkens, R.F., and Bennett, P.H.: High Prevalence of Rheumatoid Arthritis in Yakima Indians. Arthritis Rheum. 16:743-748, 1973

Henrard, J-C., and Bennett, P.H.: Étude épidemiologique de l' hyperostose vertébrale. Revue du Rhumatisme. 40:581-591, 1973

Bennett, P.H.: Some problems in the genetics and epidemiology of rheumatoid arthritis. In Dumonde, D.C.(ed.): Immunology and Infection in the Rheumatic diseases. Blackwell, London (In Press)

Serial No. NIAMDD-EFS-4(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Southwestern Indian Gallbladder Study

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: None

Cooperating Units: Indian Health Service (PHS)

Man Years:

Total:	0.3
Professional:	0.1
Other:	0.2

Project Description:

Objectives:

To determine whether cholesterol gallstones may be dissolved effectively and safely by the oral administration of bile salts.

Methods:

1. Adult Indian subjects who have asymptomatic gallstones will be identified.

2. Such subjects will be asked to participate in a double blind controlled study of the effect of oral administration of bile salts in producing the dissolution of the gallstones over a two-year period.

Major Findings:

None. All activity this year has been directed towards the further development of a detailed study protocol for the multicenter cooperative trial.

Significance of Research:

Chenodeoxycholic acid has been demonstrated to be an effective means of dissolving radiolucent gallstones in persons with a functioning gallbladder. The safety and side effects of this mode of therapy have not yet been adequately documented in a large patient series, nor in American Indians. The multicenter cooperative trial will result in such information and result in the development of the specific indications for bile salt therapy versus cholecystectomy in the treatment of cholelithiasis.

Proposed Course:

To conduct the trial in accordance with the collaborative study protocol.

Honors and Awards: None

Publications: None

Serial No. NIAMDD-EFS-5(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Prospective Study of the Complications and Outcome of Diabetic and Prediabetic Pregnancies in the Gila River Indian Community

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators: Dr. Stephen E. Dippe
Dr. Peter J. Savage
Dr. Richard F. Hamman
Dr. Max Miller

Cooperating Units: Indian Health Service (PHS)
Case Western Reserve University

Man Years:

Total:	1.0
Professional:	0.3
Others:	0.7

Project Description:

Objectives:

To determine whether pregnancies in prediabetic and/or diabetic women are associated with excessive complications during pregnancy or with excessive mortality or morbidity in the offspring.

Methods:

The course and outcome of pregnancies in all female residents of the Gila River Indian Community (Sacaton Service Unit area) will be followed and recorded in a standardized manner.

All patients in the prenatal clinic of the Sacaton Hospital receive a modified glucose tolerance test during the third trimester of their pregnancy. They receive another modified glucose tolerance test 24-48 hours post partum and are retested at approximately two year intervals in conjunction with the prospective study of diabetes mellitus in the same community.

Infants are examined to determine the presence of congenital anomalies and other characteristics, and data related to mortality and morbidity will be compiled. At age five years the children will be re-examined to detect any further abnormalities which may not have been apparent at birth. The characteristics of pregnancies will be compared among diabetic women, those prediabetic at the time of pregnancy and those women with normal carbohydrate tolerance.

Major Findings:

None. Further analysis has been deferred pending collection of additional data.

Significance of Research:

In view of the high prevalence of diabetes in the Pima Indians, studies in the prenatal clinic on this reservation provide a unique opportunity to evaluate pregnancies in prediabetics and subjects with varying degrees of carbohydrate intolerance. Retrospective studies of the course of prediabetic pregnancies have to date produced conflicting results. Although it has been established that patients with severe glucose intolerance, especially those with diabetes of long duration and significant microvascular complications, have an increased fetal and maternal mortality and morbidity, the risk in women with milder degrees of carbohydrate intolerance or uncomplicated diabetes requires further evaluation. In addition, it is not certain whether treatment will improve the outcome of those pregnancies in which there is mild glucose intolerance. Limited prospective studies of the benefit of treating pregnant women with mild glucose intolerance has so far yielded conflicting results. With the recent adoption by the Indian Health Service of more stringent criteria for the treatment of mild carbohydrate intolerance in pregnancy it is hoped that our study will be able to add to the evaluation of this problem by monitoring the impact of these therapeutic changes on the outcome of pregnancies on the reservation.

Proposed Course:

The pregnancy study will continue in conjunction with the study of the natural history of diabetes mellitus in the Gila River Indian Community. The number of pregnancies recognized to be prediabetic will increase as the mothers studied develop diabetes mellitus.

Honors and Awards:

None

Publications:

None

Serial No. NIAMDD-EFS-6(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Gila River Indian Community Autopsy Study

Previous Serial Number: Same

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Stephen E. Dippe
Dr. Joseph Likos
Dr. Richard F. Hamman
Dr. Stephen L. Aronoff
Dr. Peter J. Savage
Dr. Philip M. LeCompte
Dr. Max Miller
Dr. Delbert Eatough

Cooperating Units:
Indian Health Service (PHS)
Veterans Administration
Faulkner Hospital, Boston, Massachusetts
Case Western Reserve University
Brigham Young University

Man Years:

Total:	1.0
Professional:	0.3
Others:	0.7

Project Description:

Objectives:

1. To determine the causes of death, and associated disease processes in members of the Gila River Indian Community being followed in prospective studies on arthritis, diabetes mellitus, cholelithiasis, etc.

2. To ascertain and evaluate the histopathological changes in tissues and cells as they relate to diabetes mellitus. To assess their importance and relationship to clinical findings in members of the Gila River Indian Community upon whom autopsies are performed.

3. Evaluate factors and conditions associated with increased or decreased mortality rate in the Gila River Indians.

Research in Progress:

Renal Pathology: Review of the histologic data from 105 Pima Indian autopsies indicated that the pathology found in the kidneys of diabetic Pima was the same as in diabetics of other races.

Diabetic renal glomeruli may be larger than those from non-diabetic kidneys, but little systematic study of glomerular size or variability has occurred. Analysis was begun to compare the renal glomerular size of Pima kidneys with those of other groups as a preliminary step in biochemical analysis of collagen content and makeup.

Initial results from 8 Pima subjects are shown below:

Proportion of Renal Glomeruli Isolated in Various Sieve Fractions by Size in Microns

Approximate Glomerular size (μ)	WHITE		PIMA	
	Diabetic	Non-Diabetic	Diabetic	Non-Diabetic
	N=5	N=5	N=6	N=2
125-175	12%	47%	7%	10%
175-235	40%	47%	27%	56%
235-275	29%	4%	24%	28%
275-330	16%	1%	32%	5%
330-375	2%	0%	10%	0%
TOTAL	99%	99%	100%	99%

Trace Metals: The Pima population, with a high prevalence of diabetes, regular examinations, known diet, residence and water supply data, make them an ideal group for the evaluation of trace metal disease relationships. Dr. Delbert Eatough, Brigham Young University, Provo, Utah, has begun initial analysis of post-mortem frozen tissues from 15 patients selected at random from storage. Using atomic absorption and x-ray fluorescent spectroscopy, tissue levels of Cr, Mn, Ca, Mg, Li, Fe, Cu, Zn, and other metals will be described. The liver, pancreas, kidney, fat, hair, aorta and spleen will be analyzed from subsequent autopsies. These results will be correlated with results of glucose tolerance, electrocardiogram and other ante-mortem data.

Procedural Developments: A system to catalog all tissues stored after autopsy was developed to make possible a systematic review of these tissues in the future.

To aid in the correlation of hospital records with clinical research and autopsy data, a system is being developed for the systematic abstraction and computer storage of data from the hospital chart of patients who have died.

Significance of Research: As more subjects who have been under long term ante-mortem surveillance are autopsied, more precise clinical pathologic correlations will be available for the study of diabetes mellitus than has previously been possible.

The verification of the inter-racial similarity of diabetic nephropathy allied with work now in progress to determine renal glomerular tuft size may help confirm that the disease processes in the Pima can serve as a valid sample from which to project to all diabetics.

The Pima are the first population under continued surveillance in which trace metals concentrations have been investigated. Correlation with ante-mortem clinical data and autopsy findings may help elucidate risk factors associated with the development of diabetes, atherosclerosis and other disease entities.

Proposed Course: The systematic collection and cataloging of tissues will continue. As sufficient specimens become available, detailed examinations of various tissues will be made. Further studies of the kidney, including electron microscopy and biochemical analysis are planned.

Honors and Awards:

None

Publications:

None

Serial No. NIAMDD-EFS-7(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Coxsackie B_4 Virus Infection and its Relationship to Diabetes Mellitus

Previous Serial Number: Same

Principal Investigator: Dr. Stephen E. Dippe

Other Investigators: Dr. Peter H. Bennett
Dr. Donald Dippe
Dr. James Maynard
Dr. Ken Berquist
Dr. Max Miller
Dr. J. Deinhardt

Cooperating Units: Indian Health Service
Center for Disease Control
Case Western Reserve University
Presbyterian Hospital, Chicago

Man Years:

Total:	1.2
Professional:	0.5
Others:	0.7

Project Description:

Objectives:

1. To determine the prevalence and incidence of diabetes mellitus five years after an epidemic caused by Coxsackie B_4 virus.

2. To evaluate the relationships of serum lipids and blood pressures to diabetes and cardiovascular abnormalities.

Methods:

1. The following tests and examinations were completed on all residents of the Pribilof Islands aged 10 and above:

a. Clinical interview
b. Physical examination
c. Electrocardiogram
d. 75gm carbohydrate load tolerance test
e. Laboratory analysis for various virus studies and lipid studies.

2. All deaths occurring in the five year period are to be analyzed as to predisposing factors and cause of death.

Major Findings:

In recent years Coxsackie B_4 infection has been implicated as a possible etiologic agent in the development of diabetes mellitus. There have been both retrospective epidemiologic evaluations and animal studies which support the concept that Coxsackie B4 virus can cause hyperglycemia. A major problem for definitive study rests in the fact that the infection is very common and seldom occurs as a well defined outbreak.

Two islands in the Bering Sea, St. George and St. Paul, contain less than 500 people and are relatively isolated from the mainland. In 1967 an epidemic occurred which was identified, serologically and by virus isolation to be Coxsackie B_4. Sera were available on a large number of persons on the island prior to the epidemic. These sera were negative for Coxsackie B_4 with exception of two individuals in whom the titer was very low. Following the epidemic, 77% of the inhabitants developed significant antibody to Coxsackie B_4 virus.

An attempt was made to evaluate whether diabetes developing in the subsequent five years was related to Coxsackie B_4 infection.

Three hundred thirty-five Aleuts, 175 males and 160 females, were examined between the ages of 10 and 78 years.

Coxsackie B_4 Infection: In 1967 following the epidemic 77% of the subjects had a positive titer to Coxsackie B_4 and in the younger age groups it approached 100%. Five years later, 61% of the males and 63% of the females had detectable levels of antibody present.

Diabetes: Following a 75 gram oral carbohydrate load, 25 Aleuts were found to have a two-hour plasma glucose of 200mg/100ml and over. Only six persons could be clearly identified as having had diabetes in 1967. The remainder were diagnosed since that time. No person under the age of 25 had a two-hour plasma glucose $\geq$140mg/100ml. For those aged 25 years and over, 8% of the males, and 17% of the females had two-hour plasma glucose levels greater than 200mg/100ml.

Coxsackie B_4 Infection and its Relationship to Diabetes†

Sex	Age	Coxsackie Negative Two-hour Plasma Glucose(mg/100ml) No. Exam.	≥140 No.	≥140 %	≥200 No.	≥200 %	Coxsackie Positive Two-hour Plasma Glucose(mg/100ml) No. Exam.	≥140 No.	≥140 %	≥200 No.	≥200 %
Males											
	10-24	14	0	-	0	-	51	0	-	0	-
	25-44	17	1	6	1	6	30	3	10	0	-
	45+	36	6	17	2	6	24	10	42	4	17
Females											
	10-24	18	0	-	0	-	53	0	-	0	-
	25-44	15	6	40	2	13	30	8	27	2	7
	45+	25	8	32	3	12	16	9	56	5	31
Adjusted Total		125	-	13.3	-	5.2	204		19.7		7.8

†Excludes 6 diabetics known prior to Coxsackie outbreak

No significant association between the two hour plasma glucose level and Coxsackie B_4 neutralizing antibody titers could be demonstrated except in the 45-54 age group, where 11 of 12 persons with two hour plasma glucose levels greater than 140mg/100ml had a positive neutralizing antibody titer.

Serum Lipids: Thirty-one percent of all Aleuts had serum triglyceride levels greater than 150mg/100ml whereas 88% of diabetics (two hour plasma glucose greater than 200mg/100ml) had triglyceride levels above 150mg/100ml. Six of the ten Aleuts with triglyceride levels greater than 500mg/100ml were diabetic.

Mean serum cholesterol levels at age 10-14 were 166mg/100ml among males, 173mg/100ml in females. In both sexes, the mean serum cholesterol rose in each succeeding decade until the ages of 35-44 after which the level remained constant through the succeeding decades with the males averaging about 240mg/100ml and the females 248mg/100ml.

Significance of Research: Since these islands experienced an epidemic of Coxsackie B_4 in a previously virgin population, they presented an ideal test to establish whether or not Coxsackie B_4 is indeed a causal agent for the development of diabetes.

Five years following the epidemic no relationship between new cases of diabetes and earlier Coxsackie B_4 infection could be demonstrated. This was true in both males and females and in each age group, with the possible exception of the 45-54 age group. The significant difference found in this age group probably represents a chance finding. Previous evidence suggested that the strongest association would likely occur among juvenile onset of diabetes. We have clear evidence that no case of diabetes occurred in any person less than 25 years of age following the epidemic.

Coxsackie B_4 over a five year period did not cause the development of diabetes in this population in those aged under 25 years. Although diabetes did occur following the epidemic in the adult population, no evidence that Coxsackie B_4 infection was the cause was found.

Hypercholesterolemia and hypertriglyceridemia occurred in a large proportion of the population and as expected was more prevalent among diabetics.

Proposed Course:

In addition of an epidemic of Coxsackie B_4, the islanders suffered an epidemic of mumps occurring approximately the same time. Evaluations will be made to examine the effect of mumps on the development of diabetes. Evaluation of electrocardiographic abnormalities and lipids will be performed.

Honors and Awards:

None

Publications:

Dippe, S.E., Bennett, P.H., Miller, M.: Coxsackie B_4 Virus and Diabetes. Letters to the Editor, British Medical Journal. (In Press)

Serial No. NIAMDD-EFS-8(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Evaluation of the Prediabetic State in the Pima Indians

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Stephen L. Aronoff
Dr. Stephen E. Dippe
Dr. Richard F. Hamman
Dr. Jesse Roth
Dr. Phillip Gorden
Dr. Marvin Siperstein
Dr. Roger Unger
Dr. Joseph Williamson

Cooperating Units:
Phoenix Indian Medical Center
NIAMDD-Clinical Diabetes Section
University of California Medical School, San Francisco
University of Texas, Southwestern Medical School, Dallas
Washington University School of Medicine

Man Years:

Total:	2.0
Professional:	0.5
Other:	1.5

Project Description:

Objectives:

The natural history of diabetes mellitus has yet to be well elucidated. A prospective study of the genetically suspect prediabetic subject (defined as the non-diabetic offspring of two diabetic parents) appears to be the most direct and reliable method of describing the natural history of the disease.

Study of the prediabetic will enable us to better understand the interrelationship of hyperglycemia and microangiopathy which is presently highly controversial and poorly defined. This is possible through the study of chemical and physical abnormalities in prediabetics. Chemical abnormalities may include subtle changes in glucose and insulin as well as possible specific urinary protein abnormalities. The major physical abnormality under investigation is the muscle capillary basement membrane thickness.

Possible capillary basement membrane thickening, prior to glucose intolerance has been reported by Siperstein, but challenged by Williamson. Variation in findings in part are due to variation in techniques. Resolution of the conflict is of utmost importance in understanding the interrelationship of glucose tolerance and microangiopathy.

The purposes of the study are:

1. To study whether or not carbohydrate abnormalities, insulin abnormalities, capillary basement membrane hypertrophy and urinary protein abnormalities are characteristic of prediabetes.

2. To compare the two major techniques of basement membrane measurement.

3. To initiate a long-term prospective study of prediabetes.

Methods Employed:

Two groups have been selected for study - the non-diabetic offspring of two diabetic parents and the non-diabetic offspring of two normal parents.

The two groups are matched for age, sex and percent desirable weight and following a dietary preparation are admitted to the Clinical Research Center for testing. Tests include an oral glucose tolerance test, intravenous glucose tolerance test, cortisone glucose tolerance test, an arginine infusion, a quadriceps needle muscle biopsy, fundus photographs and a 24-hour urine collection for determination of specific urinary proteins by immuno-diffusion and characterization of volatile organic compounds.

Results:

Not yet available.

Significance of Research:

A long-term prospective study of this type will be instrumental in defining the relationship of glucose and microangiopathy. Likewise it will help to define the value of the tests being used to predict diabetes and its complications.

Proposed Course:

This study was begun in November, 1973. Approximately 40 subjects meeting the study design have been tested. Data will be collected on another 10-20 subjects prior to analysis of the data.

Honors and Awards:

None

Publications:

None

Serial No. NIAMDD-EFS-9(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Section
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Evaluation of Basement Membrane Thickness in Diabetic Pima Indians

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Stephen L. Aronoff
Dr. Stephen E. Dippe
Dr. Richard F. Hamman
Dr. Peter J. Savage
Dr. Marvin Siperstein
Dr. Joseph Williamson

Cooperating Units:
Phoenix Indian Medical Center
Sacaton Indian Health Service Hospital
University of California, San Francisco
Washington University School of Medicine

Man Years:

Total:	0.9
Professional:	0.2
Others:	0.7

Project Description:

Objectives:

Since Siperstein first reported finding thickened muscle capillary basement membranes in diabetics, multiple studies have been performed in an attempt to evaluate his results. Findings have varied remarkably from one investigator to another to the extent that while muscle capillary basement membrane thickening (MCBMT) is generally accepted as characteristic of diabetes, the frequency of its occurrence, its relationship to age, sex, duration of diabetes, and complications of diabetes is now highly controversial.

The contradictions in results appear to be related, at least in part to varying techniques of fixation and measurement employed by different investigators.

A study of MCBMT in normals and prediabetic Pima Indians is presently underway in our unit. MCBMT in diabetic Pima Indians has never been quantitated and it is not known if thickening occurs to the same extent in them as in non-Indians.

The major purposes of the study are:

1. To determine whether Pimas with diabetes develop capillary basement membrane thickening.

2. To determine whether such thickening is related to the duration of carbohydrate intolerance and to the complications of diabetes.

3. To compare capillary basement membranes of diabetic Pimas with the MCBM of normal and prediabetic Pima Indians and normal non-Indians.

Methods Employed:

Two groups of diabetics are being examined a) long-term diabetics, defined as subjects with fasting plasma glucose $\geq$140mg/100ml or a two hour post-carbohydrate load glucose $\geq$300mg% of >10 years duration with and without retinopathy and b) short-term diabetics, defined as subjects who have had a documented marked deterioration in their oral glucose tolerance over the last 5 years from a two hour post-carbohydrate load plasma glucose $\leq$140mg% to a fasting plasma glucose $\geq$140mg% or a two hour post-carbohydrate load glucose $\geq$300mg%.

Subjects will have an oral glucose tolerance test, a quadriceps needle muscle biopsy, a 24-hour urine collection for specific urinary protein determination and identification of volatile organic compounds as well as fundus photographs and direct fundus examination.

Results:

Not yet available.

Significance of Research:

This study is expected to resolve the conflict concerning the optimal method of capillary basement membrane measurement. It also will better define the relationship between the length of diabetes, diabetes complications and MCBM thickening.

Proposed Course:

Recruitment of volunteers for this study is presently underway and testing of these subjects should be concluded within six months.

Honors and Awards:

None

Publications:

None

Serial No. NIAMDD-EFS-10(c)
1. Epidemiology and Field Studies Branch
2. Southwestern Field Studies Branch
3. Phoenix, Arizona

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Evaluation of the Prediabetic State in the Pima Indians - Caucasian Controls

Previous Serial Number: None

Principal Investigator: Dr. Peter H. Bennett

Other Investigators:
Dr. Stephen L. Aronoff
Dr. Stephen E. Dippe
Dr. Richard F. Hamman
Dr. Peter J. Savage
Dr. Jesse Roth
Dr. Phillip Gorden
Dr. Marvin Siperstein
Dr. Joseph Williamson
Dr. Roger Unger

Cooperating Units:
Phoenix Indian Medical Center
University of California Medical School, San Francisco
University of Texas, Southwestern Medical School, Dallas
Washington University School of Medicine

Man Years:

Total:	0.9
Professional:	0.2
Other:	0.7

Project Description:

Objectives:

Identification of abnormalities in prediabetic individuals has been a major goal of diabetes research in attempting to understand the natural history of the disease.

Tests proclaimed as diagnostic of the prediabetic state include the cortisone glucose tolerance test, time and quantity of insulin release in relation to glucose loads and capillary basement membrane thickness.

These tests are being conducted on a group of genetically normal and genetically prediabetic Pima Indians. The very high prevalence of diabetes in the Pima people, while providing a very large group of genetically prediabetic individuals, may result in a problem of finding individuals who are truly normal. Genetically normal Pima offspring have been selected from genetically normal parents 45 years of age or older. It is conceivably possible, however, that with the high prevalence of diabetes in the tribe, virtually all the Pimas could carry the diabetic genome.

A group of non-diabetic Caucasians with non-diabetic family histories are being examined as an additional control group which will be compared with the "normal" Pimas.

Methods Employed:

The study population is selected from non-diabetic Caucasians with non-diabetic family histories who are matched in age, sex and weight with normal Pima volunteers. Each person is admitted to the Clinical Research Center for testing. Tests include an Oral Glucose Tolerance Test, Arginine Infusion, Cortisone Glucose Tolerance Test, Intravenous Glucose Tolerance Test, a Quadriceps needle muscle biopsy and a 24-hour urine collection which is analyzed for specific urinary proteins by immuno-diffusion and characterized for volatile organic compounds.

Results:

Not yet available

Significance:

The results of the tests performed on this group of normal volunteers will serve as a control group for comparison with tests in age, sex and weight matched normal and prediabetic Pima Indians.

Proposed Course:

Data collection on this study is presently underway. Results will be collected on approximately 25-30 individuals.

Honors and Awards:

None

Publications:

None

Serial No. NIAMDD-EFS-11(c)
Epidemiology & Field Studies Branch
Metabolic Diseases Epidemiology Unit
Bethesda

PHS-NIH
Individual Project Report
July 1, 1973 through June 30, 1974

Project Title: Experimental and Field Studies of Iodine Metabolism

Previous Serial Number: NIAMDD-EFS-8(c)

Principal Investigator: Robert L. Vought, M.D., M.P.H.

Other Investigators: Carol A. Mapes, Ph.D.
Freddie A. Brown, B.S.

Cooperating Units: Laboratory of Nutrition and Endocrinology, Clinical Endocrinology Branch, NIAMDD

Man Years:

Total:	3 2/3
Professional:	2 2/3
Other:	1

Project Description:

Objectives: The long-term objectives of the program are: (a) to examine closely the principal environmental factors associated with prevalence of goiter and (b) to study the nature of the metabolic defects responsible for the disease.

Methods Employed: Accomplishment of the objectives has involved bench laboratory research, clinical research and collaborative field studies. During the current year research was entirely within the laboratory.

While the *in vivo* assay described in last year's report is partially satisfactory it is tedious and difficult to apply when a large number of specimens is involved. An *in vitro* assay for goitrogens is now being developed.

Major Findings: The entire year was devoted to further purification of the goitrogen isolated in the partially purified state from broth cultures of *Escherichia coli*. This has involved: (a) production of the material in larger quantities (up to 50 liters); and (b) development of simpler *in vitro* and quantitative methods for the assay for the presence of goitrogenic compounds.

Publications:

Vought, R.L., Brown, F.A., and Sibinovic, K.H.: Antithyroid compound(s) produced by Escherichia coli: Preliminary report. J. Clin. Endocr. and Metab. (In press).

OFFICE OF SCIENTIFIC AND TECHNICAL REPORTS

Summary

Fiscal Year 1974

In a sweeping reorganization of public information activities conducted throughout the Department of Health, Education, and Welfare in FY 1974, the Office of Public Information was redesignated as the Office of Scientific and Technical Reports. This office prepares the bulk of the regular and special program reports required of the Institute by the Congress, and by the NIH and the DHEW. Its functions also include preparation of staff papers and assistance with professional publications, assistance with Congressional reports, and response to inquiries of all kinds, public and otherwise.

Reporting on the Institute's varied and complex activities, which have increased enormously in recent years, places heavy demands on the relatively small office staff. Generally speaking, this office services the general public, the professional public, the Governmental public, and the Congressional public by means of the mass media, specialized media, and by individual communications.

Scientific and Technical Reports:

The preparation of exhaustive special reports and other material in connection with Congressional appropriation hearings requires more staff man-hours than any other single project. During FY 1974 special reports were prepared on arthritis, diabetes, digestive diseases, kidney disease, hematology, cystic fibrosis and nutrition. Related reports included the Director's opening statement, the budget narrative, and highlights of research. In certain instances, such as the preparation of a special report on alcoholism, with which the research of several Institutes is concerned, collaboration with staff members of other Institutes is often required.

The "weekly report," brief abstracts of both intramural and extramural research accomplishments, forms the basis for most of the regular and special program reports required of the Institute by the Congress, by the DHEW, and by the NIH.

Typical weekly reports during FY 1974 included such diverse subjects as marked improvement in diabetic rats following "implantation" of insulin-producing pancreatic beta cells in the liver; successful preliminary clinical trials of a new drug, metiamide, which blocks gastric acid secretion and, thus, may be of value in treating peptic ulcer disease; the demonstration that prolonged treatment with gold salts beneficially alters the course of rheumatoid arthritis; production of the entire spectrum

of alcoholic liver disease in baboons by chronic administration of alcohol, thus providing an animal model for the study of such disorders, and the acquiring of further evidence in several different studies to indicate that the hormone glucagon may play an important role in diabetes.

Publications:

FY 1974 saw the issuance or revision of an unusually large number of publications of general interest. Due to public demand fact sheets were prepared on Sjogren's syndrome, ulcerative colitis, and Cooley's anemia. Several existing fact sheets, including hypoglycemia and gallstones and gallbladder disease, were updated and revised and, in one instance, an existing leaflet on systemic lupus erythematosus was converted to a fact sheet in an austerity move. In addition, a pamphlet about diabetes and one about arthritis are undergoing preparation, and the Institute's most popular publication - "Facts About Nutrition" - was revised once again and given a whole new format. More than 82,000 publications were distributed during FY 1974.

Congressional Inquiries:

The number of Congressional inquiries handled by the Office of Scientific and Technical Reports has increased spectacularly in recent years. For example, in calendar year 1963 this Office responded to 12 letter inquiries from the Congress; by contrast, in calendar year 1973 more than 150 letter inquiries were answered. Although the number of Congressional telephone inquiries also rose sharply, this type of inquiry usually is not as time-consuming as the letter type.

During FY 1974, the OSTR responded to 171 letter inquiries and 118 telephone inquiries from the Congress, a total of 289 Congressional inquiries.

Public Inquiries:

Considerable staff time is devoted to answering inquiries from the general public, which consist primarily of telephone and letter requests for information from ill persons, their concerned friends and relatives, school children engaged in science projects, practicing physicians and scientists. During FY 1974 the OSTR staff responded to 914 telephone inquiries and 885 letter inquiries, a total of 1,799 public inquiries. This figure is over and above routine requests for publications.

Press Activities:

Under the reorganization of DHEW public information activities in FY 1974 press and related activities were largely centralized within the NIH Office of Information. Nevertheless, the OSTR prepared and issued a representative number of press releases, in line with established policy to publicize the NIAMDD's research activities and programs.

Characteristic press releases included the following: (1) the awarding of an initial contract to Cedars-Sinai Medical Center, Los Angeles, to supervise and participate in a long-term study of chemical means of dissolving galistones; (2) the appointment of Dr. Thomas P. Almy to the NAAMDD Council for four years; (3) the appointment of Dr. Hans Popper to the NAAMD Council for two years, and (4) the naming of Dr. Michael Sela of the Weizmann Institute of Science to deliver the NIH Lecture in December, 1973.

During FY 1974 the OSTR responded to 70 inquiries from representatives of the various news media.

Radio and Television:

In conjunction with the NIH Office of Information a number of radio and television "spot announcements" were prepared and released concerning the availability of several NIAMDD publications. The public response to these notices has resulted in a backlog of several thousand requests for Institute publications.

Exhibits:

The Office of Scientific and Technical Reports also conducts the NIAMDD program of scientific exhibits, a prime source of professional education. At the request of various scientific organizations, NIAMDD displayed four different exhibits during FY 1974 at 7 medical and scientific meetings throughout the country, which were viewed by approximately 50,000 physicians, scientists and paramedical people. Institute exhibits on human growth hormone, the artificial kidney, gout, and cystic fibrosis were among those shown. Institute publications and reprints are distributed in conjunction with these exhibits.

OFFICE OF THE DIRECTOR

Summary

Fiscal Year 1974

Dr. G. Donald Whedon, Director
Dr. Ronald W. Lamont-Havers, Deputy Director
Mr. F. L. Mills, Executive Officer

The office of the Director has overall responsibility for directing and coordinating basic laboratory research; clinical investigations; extramural activities such as research grants, research fellowships, and training grants; contract research and developmental programs; and epidemiologic and clinical field studies. In addition, this office cooperates with other scientific organizations in coordinating medical research in the Institute's fields of interest; cooperates and maintains liaison with other Institutes and constituents of other Federal agencies; and participates in determining policy governing the National Institutes of Health. It is also responsible for the collection and dissemination of informational material to interested professional and lay individuals and groups.

During this year Congress indicated an interest in having the National Institutes of Health form an Inter-Institute Research Coordinating Committee to reflect NIH-wide efforts in diabetes research. In view of the primary interest of this Institute in the area of diabetes, Dr. G. Donald Whedon, Director, NIAMDD, was appointed to serve as Chairman and the Diabetes Program Director of NIAMDD was appointed to serve as Executive Secretary of the group.

As a result of this Congressional interest in Diabetes the Institute has become heavily involved in strengthening the Diabetes Program in anticipation of pending legislation which will add specific responsibility to the Institute, such as the appointment of an Associate Director and related staff for Diabetes, Endocrinology and Metabolism.

Congress is also reviewing legislation to strengthen the Diabetes Program by establishing a Diabetes Study Commission and the Institute will undoubtedly be assigned the Executive Secretariat of the Commission. This will require the Institute to become heavily involved in the administrative role of the Commission.

The Institute is also aware of impending legislation in the Senate and House relating to the support of Arthritis and allied conditions. These bills are similar to the Diabetes bill which will direct the Institute to become more heavily involved in expanding the Arthritis program.

The reorganization of public information activities at the National Institutes of Health resulted in the abolishment of the Office of Public Information and the establishment of the Office of Scientific and Technical Reports.

A new Diabetes Branch was created within the Clinical Investigations area of NIAMDD to reflect more adequately the interest of the Congress and this Institute in Diabetes. Dr. Jesse Roth was appointed the Chief of the Branch.

The realignment of some of the clinical sections within the Institute to better reflect the scope of functions and responsibilities of the area resulted in renaming the Digestive and Hereditary Diseases Branch to the Digestive Diseases Branch. A new section on Gastroenterology was established and three sections were transferred from the Metabolic Diseases Branch.

A section on Chemical Immunology was established in the Arthritis and Rheumatism Branch. Dr. Henry Metzger was appointed Chief of the Section.

A new section on Macromolecular Biology was established in the Laboratory of Chemical Biology and Dr. Alan N. Schechter was appointed Chief of the Section.

The Section on Oxidation Mechanisms was established in the Laboratory of Chemistry. Dr. Donald M. Jerina was appointed Chief.

Sections that were abolished during the year were: Grants Operation Section and the Administrative Services Section of the Grants Management Branch, Office of the Associate Director for Extramural Program Activities; the Section on Biosynthesis and Control, Laboratory of Chemical Biology; the Section on Electrochemistry and Colloid Physics, Laboratory of Chemical Physics; the Section on Rheumatic Diseases, Laboratory of Experimental Pathology, and the Section on Steroids, Laboratory of Chemistry.

Several new appointments have been made to key positions within the Institute this year. Dr. Phillip Gorden was appointed Clinical Director. Dr. Robert G. Martin was appointed Acting Chief of the Laboratory of Molecular Biology. Dr. Elizabeth S. Maxwell was appointed Acting Chief, Section on Metabolic Enzymes, Laboratory of Molecular Biology. Dr. Robert T. Simpson was appointed Chief, Section on Developmental Biochemistry, Laboratory of Nutrition and Endocrinology. Dr. Gerald Aurbach was appointed Chief of the Metabolic Diseases Branch. Dr. Cornelis P. J. Glaudemans was appointed Acting Chief, Section on Carbohydrates and Dr. Richard J. Podolsky was appointed Chief, Laboratory of Physical Biology.

The activities of the Office of Scientific and Technical Reports are included in a separate report which precedes this summary. Efforts continue to provide an integrated program of scientific technical information and reports related to the Institute's program. Responses to 1799 public inquiries were made during the year in addition to 289 Congressional inquiries which were made by this office. Over 82,000 publications of general interest were distributed.

Volume of contract activity continued at a relatively level rate with a total obligation for the year approximating $6,338,712,000. However, two members of a seven-member staff were lost at the end of F.Y. 1973, and they were not replaced during F.Y. 1974. Artificial Kidney, Hormone

Distribution, Scientific Communications, and augmentation of intramural research remained about the same. The clinical study of treatment of Crohn's Disease continued and was augmented by an additional obligation of $590,000 necessitated by expanding the number of participating clinical centers from 8 to 15. The contract, awarded in June 1973, for a clinical study of the dissolution of gallstones by bile acid feeding, was nurtured through its first year of operation. No major problems developed, but some administrative and other procedural rough edges had to be smoothed over. It was necessary to provide for an unanticipated additional funding of approximately $160,000 which was dictated by increased demands of the F.D.A. for more intensive animal toxicity testing. The second increment of funding also was obligated to enable initiation of Phase II of the study (beginning of the clinical trials). A new contract was awarded for developing and testing an iron-chelating agent for treatment of Cooley's Anemia. Two other projects were started by issuing requests for proposals (no contract award was anticipated prior to F.Y. 1975). These projects were for research and development in gastrointestinal endoscopy and for diagnosis and treatment of psoriasis, with the latter having a potential for expanding into a multi-center clinical trial.

The Basic Ideas in Biology and the Basic Ideas in Chemistry courses have continued to be successful. The biology class includes such topics as laboratory math, physiology, anatomy, microbiology, and animal care. Topics in the chemistry class include analytical procedures, characteristics of various classes of compounds, and biochemical reactions. In June the second semester of biology and the third semester of chemistry will be completed. These courses are designed to provide a basic knowledge of these disciplines to those working in laboratories who have little formal training in the sciences. The emphasis is on skills acquisition in a laboratory setting with theoretical material minimized. The material presented is geared to the research interests of the NIH. To allow as much practical experience as possible, class size is limited to 6 students in each of 2 sections for each course. Supervisors are encouraged to utilize the new skills acquired by their employees. Interest in the classes is high as evidenced by the low dropout rate and the willingness of supervisors for their employees to participate. During the year, short-term training opportunities were arranged for 61 secretarial-clerical employees, 13 supervisory managerial personnel and 111 members of the professional staff.

Equal Employment Opportunity

Mr. J. Harrison Ager has been appointed as NIAMDD EEO Coordinator on a full-time basis. Mr. Ager has also been appointed as an NIH EEO Counselor and has received appropriate training. He has as a major function of his position the conduct of recruiting activities in order to bring more minority professionals to this Institute as well as minorities at other levels. The appointment of Mr. Ager has been of considerable assistance in the enhancement of our EEO program.

A series of meetings were held with all supervisors within NIAMDD to emphasize training, recruitment, counseling and various policies regarding

better understanding and management of employees. The supervisor's role in the NIAMDD Affirmative Action Plan was discussed. Individual Laboratory and Branch EEO meetings are continuing.

The NIAMDD EEO Advisory Committee, chaired by the Director, continues to meet on a regular basis to discuss all matters relating to the Affirmative Action Plan of this Institute and the sub-Committees continue to be active. One of the goals for the coming year is to update our Affirmative Action Plan. We are also in the process of developing an EEO awards program.

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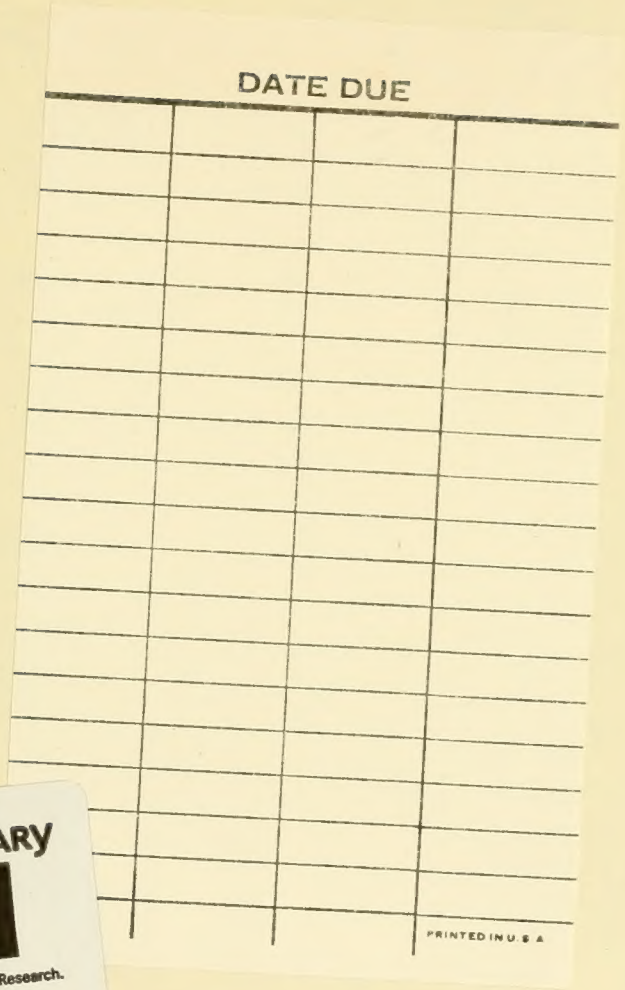

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